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Intracellular lipids in *Bos indicus* and *Bos taurus* oocytes

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INTRACELLULAR LIPIDS IN *BOS INDICUS* AND *BOS TAURUS* OOCYTES

A Thesis

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
Requirements for the degree of
Master of Science**

in

**The Interdepartmental Program in
Animal and Dairy Sciences**

by

**Casey Bryant Ballard
B.S., Texas A&M University, 2004
May 2007**

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ABBREVIATIONS

ATP	Adenosine Triphosphate
BB	Belgian Blue
BCS	Body Condition Score
BSA	Bovine Serum Albumin
BSE	Bovine Spongiform Encephalopathy
CHOL	Cholesterol
CIDR.....	Controlled Intravaginal Releasing Device
CO ₂	Carbon Dioxide
COCs.....	Cumulus Oocyte Complexes
CPAs	Cryoprotective Agents
DMSO.....	Dimethylsulfoxide
E ₂	Estradiol
eCG	Equine Chorionic Gonadotropin
EG	Ethylene Glycol
ET	Embryo Transfer
FA	Fatty Acid
FCS	Fetal Calf Serum
FMD	Foot and Mouth Disease
FSH	Follicle Stimulating Hormone
GC	Gas Chromatography
Gly	Glycerol
hCG	Human Chorionic Gonadotropin
HF	Holstein Friesian
ID	Identification Number
IM	Intra-Muscular
IVF.....	<i>In Vitro</i> Fertilization
IVM.....	<i>In Vitro</i> Matured
IVP	<i>In Vitro</i> Produced
LH.....	Luteinizing Hormone
M-II	Metaphase-II
NRU	Nile Red Units
OCM.....	Oocyte Collection Medium
PBS	Phosphate-Buffered Saline
PL.....	Phospholipid
PROH	Propamediol
PVP	Polyvinylpyrrolidone
RPM	Rate Per Minute
TAG	Triacylglycerol
TCM-199	Tissue Culture Medium-199
TEM.....	Transmission Electron Microscopy
TUGA	Transvaginal Ultrasound-Guided Aspiration

ABSTRACT

It has been proposed that *Bos indicus* when compared with *Bos taurus* embryos are more sensitive to cryopreservation procedures due to higher intracellular lipid content of the embryos. With cryopreservation of *Bos indicus* embryos increasing for export, it is essential to improve the viability of these cryopreserved embryos. In Experiment I, M-II oocytes from mice, pigs and cows were subjected to two different procedures to evaluate lipid content. The lipid content present in pig oocytes was significantly ($P<0.05$) higher when compared with mouse and cow oocytes. Pig oocytes had a lower settling point in the sucrose buoyant density gradient and higher Nile Red units, which indicated higher lipids present in the oocytes. In Experiment II, it was determined that M-II oocytes aspirated from superstimulated Brahman donors were significantly higher ($P<0.05$) in lipid content compared with English breed donors. The lipid content present in Brahman oocytes was higher when compared with English breeds when tested with a sucrose buoyant density gradient and Nile Red staining. In addition, donor females with higher body condition scores were significantly ($P<0.05$) higher in oocyte lipid content when compared with donor females of lower body condition scores. Furthermore, animal body condition scores, cholesterol and triglyceride levels prior to oocyte aspiration may be indicators of intracellular lipids in the oocytes and embryos. To our knowledge, this is the first report that evaluated intracellular lipid content of M-II oocytes of different beef breed types using a sucrose buoyant density gradient and Nile Red staining.

CHAPTER I

INTRODUCTION

Intracellular lipids present in embryos are a major factor that may hinder the cryopreservation of valuable embryos produced from superstimulated bovine donors. Research has been conducted in different species to try to fully understand lipid reserves and their utilization in both the oocyte and embryo. However, lipids have many functions in the embryo development process. In addition, valuable research is needed to fully understand the involvement of these lipids found in oocytes and embryos. Kim et al. (2001) stated that lipids play a significant role in energy storage, cell structure and modifying both the physical properties and metabolic function of biological membranes. Research evaluating intracellular lipids indicates that lipids present in embryos may be a limiting factor of improving cryopreservation (Nagashima et al., 1994; Leibo et al., 1995).

Porcine oocytes are rich in lipid content when compared with bovine oocytes. This may be associated with the inability of porcine oocytes and embryos to be cryopreserved successfully (McEvoy et al., 2000). Of the fatty acids present in the oocyte, triglycerides are the major component found within intracellular lipids (Homa et al., 1986), providing a large potential energy reserve and building materials for the cytoplasmic membrane of future embryos (Isachenko et al., 1998). Studies have indicated that the high intracellular lipid content present in the oocyte and embryo may be associated with the relatively low ability to cryopreserve these genetic sources (Wilmut, 1972, Polge et al., 1974; Nagashima et al., 1992). Nagashima et al. (1994;1995), Dobrinsky (2001); Park et al. (2005) demonstrated that the tolerance of porcine embryos to cryopreservation can be increased after decreasing their relatively high content of cytoplasmic lipid droplets by aspirating the lipids. These manipulated embryos continue to develop to the blastocyst stage *in vitro* and produce live

offspring *in vivo*. Understanding the functions of lipids and how they are involved in oocyte and embryo development could improve cryopreservation techniques with bovine embryos used in embryo transfer (ET).

Chilling alone does not appear to compromise the embryos integrity, but subtle changes in membrane structure and function may occur due to thermotropic phase transition of polar lipids and consequent lateral phase separation of membrane components (Didion et al., 1990). Bovine oocytes and embryos are especially sensitive to chilling injury (Balasubramanian et al., 2006) and intracellular lipid content of the oocytes and embryos influence their sensitivity to chilling (Pangestu et al., 1996).

Comprehensive studies from commercial ET operations have recorded a number of factors related to embryo transfer of both fresh and frozen embryos (Looney et al., 1996, Arreseigor et al., 1998, Hasler, 2001, Visintin et al., 2002; Steel et al., 2004). Studies evaluating the cryopreservation of embryos have evaluated different factors to improve cryopreservation procedures.

It has been proposed that *Bos indicus* embryos when compared with *Bos taurus* embryos do not survive cyropreservation well due to the amount of intracellular lipids present in the embryo. It is essential to improve the viability of these cryopreserved embryos in order to increase pregnancy rates and to decrease the high cost associated with multiple embryo transfers.

Many observations on embryos between *Bos taurus* and *Bos indicus* report pregnancy rates after frozen-thawed transfers (Visintin et al., 2002; Steel et al., 2004). Evaluating the lipid content in oocytes between the 2 different breeds has not been reported. The main objective of this study is to assess if intracellular lipid content in Metaphase-II (M-II) stage Brahman oocytes are higher in lipid composition when compared with English breed

M-II stage oocytes. The comparison of lipids in oocytes will be tested using a buoyant density gradient and Nile Red staining, which has been proven to identify lipids in mammalian oocytes (Genicot et al., 2005).

CHAPTER II

LITERATURE REVIEW

CYRYOPRESERVATION OF EMBRYOS

Genetic improvement in beef and dairy herds has been enhanced because of the ability to cryopreserve embryos. This is clearly illustrated by the large number of frozen embryo transferred each year. In 1990, more than 500,000 bovine embryos were either transferred or frozen (Shaw et al., 2000; Thibier, 2000). Bovine oocytes and embryos are considerably more cryotolerant than porcine oocytes and embryos (Isachenko et al., 2001). Two major categories of cryoprotectants have been used for cryopreservation of embryos: penetrating cryoprotectants (e.g, glycerol, dimethylsulfoxide [DMSO], 1, 2-propanediol [PROH], ethanol) and nonpenetrating cryoprotectants (e.g, polyvinyl-pyrrolidone [PVP], sucrose and glucose) (Niemann, 1991). Cryoprotective agents (CPAs) are needed at molar concentrations to prevent cold shock of freezing tissues and cells. However, CPAs may also cause cell damage due to osmotic effects or chemical toxicity (Parks et al., 1992). Mammalian oocytes, embryos and *in vitro*-produced embryos exposed to cryoprotectants are sensitive to low temperatures close to 0°C without freezing (Leibo et al., 1995; Martino et al., 1996; Seidel, 2006). The embryo temperature sensitivity may be due to lipids found in embryos, which is thought to influence sensitivity to cooling and freezing (Vajta et al., 2006). The addition of cryoprotectant to embryos is required to avoid cell damage to embryos during the freezing-thawing steps.

Embryos frozen usually produce lower pregnancy rates when compared with fresh transferred embryos (63.4% and 72.9%, respectively) (Hasler, 2001; Massip, 2001). Bovine embryo quality also effects cryopreservation survival rate (Looney et al., 1996). High grade cryopreserved embryos usually have a better pregnancy rate than embryos of lower grades.

Although, pregnancy rates from cryopreserved embryos are lower when compared with fresh embryos, it has been suggested that the low pregnancy rates are associated with embryo intracellular lipids, which cause cell damage during cryopreservation (Hasler, 2001; Seidel, 2006). Recently, vitrification has resurfaced as a way to cryopreserve mammalian gametes. This method has improved bovine pregnancy rates by modifying the lipid phase transition temperature, which consequently alters the chilling sensitivity (Horvath and Siedel, 2006).

Bovine oocytes and embryos are particularly difficult to cryopreserve successfully due to their structural sensitivity to cooling and freezing which results in low blastocyst survival from embryos cultured *in vitro* (Seidel, 2006). When cells are frozen, they are subjected to increased stress resulting from water-solute interactions that arise through ice crystallization (Dobrinisky, 1996; Visintin et al., 2002). The process of cryopreservation involves exposure to low temperatures even before freezing occurs, which starts the formation of ice crystals (Shaw et al., 2000).

This process is known to induce changes in two dimensional membrane lipid organization or “packing” (lipid phase transitions) and, in turn, modifies the kinetic properties of intramembranous enzymes (Visintin et al., 2002; Muldrew et al., 2004). The crystallization occurs when embryos are cooled to temperatures of -50°C, which induces unfrozen pockets of hyperosmotic solution (Holt, 2000). The negative effect on cell homeostasis results in a withdrawal of intracellular water, subsequent cell shrinkage and influx of ions (Mazur et al., 1986; Visintin et al., 2002). The thawing of embryos is the reversal of embryo freezing, and this causes a consequent inward water flux, which disrupts or lyses the cell membranes (Mazur et al., 1986; Dobrinisky, 1996). The process of rapid cryopreservation causes lethal intracellular ice formation. The optimal cooling rate is thought to be slow enough to prevent this lethal effect, yet fast enough to minimize the harmful effects of prolonged exposure to

high salt concentrations (Visintin et al., 2002). Efforts to correlate susceptibility to cryo-injury with the membrane lipid composition in porcine and bovine embryos have suggested that cold shock is more severe when membrane sterol concentrations are low and polyunsaturated fatty acid concentrations are high (Visintin et al., 2002).

Bovine and ovine oocytes are sensitive to low temperatures (Leibo et al., 1995). The high intracellular lipid content found in the oocytes among different species is linked to their low survival rate (McEvoy et al., 2000). However, among porcine, bovine and ovine oocytes, the porcine oocytes are the least tolerant, possibly due to their relatively high lipid content (Men et al., 2006). Wilmot (1972) reported that 18-cell porcine embryos cooled to +15°C resulted in 84% survival rate but none of the embryos survived when cooled at +10°C or below. The low survival rate of embryos being cooled has also been shown in bovine embryos (Wilmot et al., 1975, Mohr et al., 1981, Looney et al., 1989). Polge et al. (1974) and Pollard et al. (1994) reported that 8-cell to 16-cell bovine embryos lysed when temperatures reached 0°C due to their sensitivity; whereas, sheep embryos at the same morphological stage survived at this temperature. However, bovine late morula stage embryos that were cooled at the same temperatures survived and developed into normal calves after being transferred (Pollard et al., 1994). The low survival rate of chilled or cryopreserved embryos after transfer severely limits the chance of pregnancy in pigs (Nagashima et al., 1988).

Studies have suggested that the sensitivity of porcine embryos to low temperatures may be related to their relatively high lipid content or composition and have focused on improving porcine embryo cryopreservation (Nagashima et al., 1994). Niemann (1991) found that the zona pellucida of porcine embryos can tolerate the osmotic alterations occurring after the addition and removal of cryoprotectants, such as glycerol, DMSO or sucrose. Porcine morula stage embryos, however, have little or no survival after cryopreservation due

to the high amount of lipid found in the embryos (Nagashima et al., 1989; Nagashima et al., 1992). Additionally, porcine embryos from the early cleavage stage to the blastocyst stage are known to be highly sensitive and start to degenerate when cooled below 15°C with survival after exposure to lower temperatures being confined to embryos from the pre-hatching stages (Wilmut, 1972; Polge et al., 1974; Nagashima et al., 1994).

Severe damage of the intracellular lipid droplets occurring after cooling in the porcine embryos has been observed by electron microscopy (Polge, 1978). The lipid droplets in porcine embryos are abundant at the 2-cell to 8-cell stage embryos and then decline markedly at the blastocyst stage (Niimura et al., 1980). The limited success rate at the blastocyst stage has been proposed to be due to the high content of intracellular lipids (Nagashima et al., 1988). The lipid droplets in the cytoplasm generally have a close spatial relationship with the smooth endoplasmic reticulum in the embryo (Nagashima et al., 1994). Uneven intracellular ice crystal formation starts to form when porcine embryos are cooled, which may explain why porcine oocytes and embryos have a poor tolerance to cryopreservation (Nagashima et al., 1988). No clear explanation has been reported of what role the lipids play in the developing embryo, except that the embryo apparently needs the lipids for energy storage and development. However, high lipids in porcine embryos hinder post-cryopreservation viability (Homa et al., 1986).

It has been documented that porcine embryos at the expanded and hatched blastocyst stages have a higher tolerance to cooling compared with morulae and early blastocysts (Nagashima et al., 1988). Live births of piglets have been recorded from embryos that were frozen in 1.5 M glycerol, thawed at 35°C and transferred into recipients (Hayashi et al., 1989; Kameyama et al., 1990; Kashiwazaki et al., 1991). It has been shown that porcine embryos frozen in liquid nitrogen have had survival rates between 17% and 50%

for expanded blastocysts and between 38% and 96% for hatching and hatched blastocysts (Nagashima et al., 1994). However, pre-morulae and morulae did not survive cryopreservation, which indicates the peak for preserving porcine embryos is at the hatching stage (Nagashima et al., 1994). Porcine embryos at the blastocyst stage were able to survive cryopreservation due to the low lipid content compared with morula stage embryos (Niimura et al., 1980). Beyond the blastocyst stage, survival rate of the embryos decreased. As porcine embryos develop, the lipid content starts to decline, which may be the reason for the post-cryopreservation survival rate of porcine embryos at the blastocyst stage (Nagashima et al., 1988).

The developing bovine embryos follow the same pattern as the porcine embryo. As the bovine embryos develop, the intracellular lipid content declines (Mohr et al., 1981). At the early cleavage stage, bovine embryos contain a large number of lipid droplets (Mohr et al., 1981) that decline at the blastocyst stage, which coincides with a loss in sensitivity to cooling (Trounson et al., 1976a). Bovine morula stage embryos cooled to 7.5°, 7° and 0°C for 24 hours have survived after being warmed, although the percentage of normal embryos decreased with increasing time at the lower temperatures (Trounson et al., 1976a). Bovine blastocysts are resistant to cooling when compared with morula stage embryos, which is likely associated to the lower lipid content of day-7 embryos (Trounson, 1976a). Additionally, 66% of the blastocyst stage embryos survived cooling at 0°C for 24 hours, and 50% of them survived even after being cooled for 48 hours (Trounson et al., 1976b). Very few 8-cell to 16-cell stage bovine embryos survived cooling at 0°C.

FATTY COMPOSITION OF OOCYTES

Cryopreservation of oocytes and embryos is a useful tool for the storage of valuable genetic material (Dobrinsky, 1996). Frozen-thawed embryo transfers are widely used to

improve cattle genetics of specific lines (Hasler, 2003). However, lower post-thaw survival rates of cryopreserved embryos can reduce the use of embryo transfer as an assisted reproduction technique. Chilling to -25°C alone does not appear to compromise bovine embryo integrity, nevertheless the subtle changes in membrane structure and function may occur due to thermotropic phase transition of polar lipids and consequently lateral phase separation of membrane components (Parks et al., 1992). Watson et al. (1987) showed that the subtle changes in membrane were associated with shock or cold shock. This shock causes lysis of the cells and renders the embryos to fully develop after cryopreservation.

The presence of intracellular lipids in bovine oocytes and embryos has been shown to account, at least partially, for the low post-cryopreservation tolerance (Diez et al., 1996; Dobrinsky et al., 1996). Lipids that are present in oocytes may have a multitude of functions in the development of embryos (McEvoy et al., 2000). Kim et al. (2001) demonstrated that lipids in bovine embryos play a significant role in energy storage, cell structure and in modifying the physical properties and metabolic function of biological membrane. Research has been conducted in different mammalian species to understand lipid reserves and their uptake and utilization by the oocytes and embryo, since intracellular lipids in the oocytes and embryos may be a limiting factor during cryopreservation (Diez et al., 1996, Dobrinsky et al., 1996, Otoi et al., 1997; McEvoy et al., 2000). Research on intracellular lipids and their effect on cryopreservation in terms of quantitative and qualitative analysis in bovine embryos have been analyzed (Kim et al., 2001).

Studies have been conducted to determine the metabolism, type and amount of lipids present in bovine, porcine and ovine oocytes and embryos (McEvoy et al., 1997; Ferguson et al., 1999). It has been shown that bovine embryos metabolize glycogen, protein and lipids from the zygote to the blastocyst stages (Partridge et al., 1996; Thompson et al., 1996). Of

the lipids present, triglycerides have been reported to be the most abundant in bovine and porcine embryos (Hillman et al., 1980, Young et al., 1994, Coull et al., 1997; McEvoy et al., 2000).

Bovine, porcine and ovine oocytes evaluated by gas chromatography to determine fatty acid mass composition, are primarily phospholipids and triglycerides (McEvoy et al., 2000). The fatty acid composition present in porcine oocytes was reported to be ~2.5-fold higher compared with bovine and ovine oocytes (McEvoy et al., 2000). When fatty acids were broken down by type, triglycerides were the highest content and phospholipids were next, which, the triglyceride accounted for 25% of total lipids in porcine, bovine and ovine oocytes. Triglyceride content was also found to be the most abundant lipid present in *in vivo* and *in vitro*-produced bovine embryos. The triglyceride levels of porcine oocytes are higher than bovine and ovine oocytes (McEvoy et al., 2000). However, there is no significant difference in fatty acid composition or quantity between bovine and ovine oocytes. The high triglycerides found in porcine, bovine and ovine oocytes may underlie the contrasting chilling sensitivities of porcine and bovine embryos (Diez et al., 1996, Dobrinsky, 1996; McEvoy et al., 2000).

Homa et al. (1986) provided the first detailed lipid analysis of immature pig oocytes. Large masses of immature porcine oocytes were subjected to conventional extraction and gas chromatographic techniques to evaluate the different type of lipids present in the oocytes. Triacylglycerol (100.71 nmol/mg) followed by cholesterol (32.71 nmol/mg) were the major lipid components found in the porcine oocytes. Additionally, Guraya (1965) found a high content of triglycerides and cholesterol in cat and dog oocytes, with triglycerides being in the highest concentration. It has been hypothesized that triglyceride and cholesterol was present in porcine oocytes, like in murine oocytes, for specialized metabolic requirements of

the cell, which promotes oocyte development for maturation process (Loewenstein et al., 1964; Leese, 1991).

Ferguson et al. (1999), using fluorescence microscopy, has quantified triglyceride content of bovine oocytes and embryos produced *in vitro* in presence or absence of fetal calf serum (FCS). The mean triglyceride content present in immature oocytes was 59 ± 1.37 nmol/mg before maturation compared with 46 ± 0.85 nmol/mg after incubation for 24 hours in maturation medium supplemented with FCS. For embryos also cultured in presence of serum, the triglyceride levels decreased to 34 ± 0.92 nmol/mg at the 2-cell stage and increased from the 8-cell stage up to hatched blastocyst stage (62 ± 1.14 nmol/mg). However, embryos cultured in the absence of serum, showed no changes in triglyceride content from the 2-cell up to the blastocyst stage (33 nmol/mg).

The triglyceride content found in embryos cultured in serum-free medium does not differ from *in vivo*-derived embryos collected on day 7 (estrus = day 0) (33 ± 0.70 ng/oocyte). In contrast, the triglyceride concentration of embryos grown in FCS increased steadily from the 8-cell to 16-cell stage to a value in hatched blastocyst. The value for embryos grown in serum added medium doubled in triglycerides when compared with embryos grown in serum free medium. The decrease in triglycerides at maturation and up to the 8-cell stage embryo may indicate a metabolic role in the oocyte, which suggests that lipids are used as in energy source during embryo development (Fleming et al., 1972, Kruip et al., 1983, Homa et al., 1986; Isachenko et al., 1998).

Serum is a common constituent of embryo culture media for *in vitro*-produced embryos (Rizos et al., 2003). Embryos developed in a serum-supplemented medium, showed morphological differences, such as abundant intracellular lipid droplets (Thompson et al., 1995; Abe et al., 1999). Using gas chromatography, Kim et al. (2001) evaluated the

lipid content and fatty acid composition present in fresh immature and *in vitro*-matured bovine oocytes cultured in medium supplemented with serum or without serum, and also those of frozen–thawed immature oocytes. Triglycerides were the most abundant fatty acid present in both immature and *in vitro*-matured oocytes using medium containing FCS (57.6 pmol/oocyte). However, triglyceride concentrations of oocytes that were matured in medium supplemented with FCS were lower in triglyceride content than oocytes matured with no serum in the medium (36.6 vs. 27.7 pmol, respectively). The decrease in triglycerides varied among oocytes depending on the presence of FCS or serum-free medium. Additionally, triglycerides and total cholesterol were lower when comparing *in vitro*-matured oocytes with immature oocytes. The lipid content present in the oocyte are used for energy source and maturation (Lehninger, 1972). However, lipids may be incorporated into the oocytes cytoplasm during the *in vitro* maturation in the presence of FCS (Kim et al., 2001).

Of the farm animals, oocytes and embryos from pigs are less cryotolerant than bovine and ovine oocytes and embryos (McEvoy et al., 2000). Such differences may be associated with the high intracellular lipids present in the porcine oocytes and embryos (Nagashima et al., 1988, Nagashima et al., 1994, Young et al., 1994; McEvoy et al., 2000). Porcine oocytes and embryos are darker, within the ooplasm, than ovine and bovine oocytes and embryos, which indicates a higher quantity of endogenous lipids (Sturmey et al., 2003). McEvoy et al. (2000) showed that the immature porcine oocyte contains higher intracellular lipids than murine, bovine and ovine oocytes (156 vs. 4, 138 and 89 nmol/mg, respectively).

Triglycerides are a major component of intracellular lipid in mammalian oocytes and it is suggested that the lipids provide a large potential of energy reserve for the development of embryos (Sturmey et al., 2003). These researchers evaluated the role of different energy sources of porcine oocytes and embryos cultured *in vitro*. The triglyceride content was

measured as well as oxygen and glucose consumption, to determine actual adenosine triphosphate (ATP) production. Porcine oocytes contained 135 ± 4.9 ng/oocyte of triglyceride, which decreased to 122.5 ng/oocyte during *in vitro* maturation. Additionally, 117.7 ng/oocyte of triglyceride were detected during embryo development without any changes in triglyceride concentration. Oxygen consumption in the oocytes and embryos throughout the experiment were low; however, oxygen consumption reached a peak during the blastocyst stage. The results indicated that endogenous triglycerides found in porcine oocytes may be used as an energy source during *in vitro* maturation and that most of the ATP (9% to 97%) produced during embryo development comes from oxidative phosphorylation (Sturmey et al., 2003).

CRYOTOLERANCE DIFFERENCE OF OOCYTES BETWEEN CATTLE BREEDS

Differences in reproductive efficiency between *Bos indicus* and *Bos taurus* have been reported (Chenoweth et al., 1994; Abeygunawardena et al., 2004). *Bos indicus* and *Bos indicus* crossbred cattle females have a higher production of milk with higher butterfat content than *Bos taurus* females. Gestation length of Brahman is significantly longer than European or Continental breed (292 vs. 285 days, respectively). Additionally, *Bos indicus* cattle exhibit a shorter and less intense estrous cycle and a later onset of puberty than *Bos taurus* breed females (Reynolds et al., 1963). It has been shown, that Brahman embryos when collected and frozen using industry standard protocols produce lower pregnancy rates compared with English or Continental breeds (Looney, unpublished data). Moreover, pregnancy rates from cryopreserved bovine embryos have been found to be different among *Bos indicus* and *Bos taurus* breeds. These observations appear to correlate with cytoplasmic lipid content in the embryos. In general, it has been accepted that bovine embryos with larger lipid droplets have reduced cryotolerance at the morula stage (Seidel, 2006). Pregnancy rates after transferring frozen bovine embryos among different breeds

have been reported that shows a difference between *Bos indicus* and *Bos taurus* cattle (Visintin et al., 2002; Steel et al., 2004). However, the quantification of embryo lipid content among different cattle breeds have not been reported.

Many factors affect pregnancy rates from frozen-thawed transferred bovine embryos. Quality grade, stage and cryoprotectant used during the cryopreservation procedure effect pregnancy rates (Looney et al., 1996). Hasler (2001) has reported the effect of embryo grade on pregnancy rates from frozen and thawed embryos in glycerol and ethylene glycol. Embryos with grades 1, 2 and 3 resulted in 57.1, 52.9 and 32.1% pregnancy rates, respectively. In addition, embryos frozen in either ethylene glycol or glycerol from Holstein cows resulted in lower pregnancy rates compared with embryos from beef cows. Embryos cryopreserved from *Bos indicus* or *Bos taurus* have also shown to be different when frozen in different cryoprotectants (Arreseigor et al., 1998; Looney, unpublished date). Embryos collected from *Bos indicus* donors showed a lower pregnancy rates after cryopreservation in ethylene glycol than in glycerol (15.4% and 32.9% $P < 0.01$, respectively), however, *Bos indicus* embryos frozen in glycerol and ethylene glycol were lower than that of similarly treated *Bos taurus* embryos (Arreseigor et al., 1998). Also, lipid droplets have been found to be larger and in greater number based on observations in the *Bos indicus* than in *Bos taurus* blastocysts stage embryos (Visintin et al., 2002).

The lower pregnancy rates of post-thawed embryos from *Bos indicus* and *Bos taurus* are thought to be characteristic of pregnancy rates found in Jersey and Holstein cattle (Steel et al., 2004). Jersey embryos frozen in glycerol and ethylene glycol produced lower pregnancy rates than Holstein embryos. Pregnancy rates using fresh, glycerol and ethylene glycol frozen embryos were lower in Jersey than in Holstein donors. Although the amount of lipid droplets in the two breeds was not evaluated, there was a significantly higher pregnancy

rates from frozen-thawed embryos produced from Holstein when compared with frozen-thawed embryos collected from Jersey donors. When evaluating pregnancy rates from fresh transferred embryos collected from Jersey and Holstein donors, there was no significant difference. Many observations on cryopreservation of embryos between *Bos taurus* and *Bos indicus* are based on pregnancy rates after transferring frozen-thawed embryos, however, little scientific literature on the intracellular lipid content between *Bos indicus* and *Bos taurus*.

LIPID LEVELS IN BLOOD AND EMBRYOS

Nutritional management as well as environment conditions are major factors controlling reproduction in beef cattle. Environment (e.g, temperature, weather, location, feed and forage) can produce profound effects on ovarian and fertility function in beef cattle, which may be a result of altered circulating serum levels of cholesterol and triglycerides (Vizcarra et al., 1998; Adamiak et al., 2005). Metabolic changes in the blood serum due to environmental changes may be reflected in the biochemical composition of follicular fluid, which can directly or indirectly affect the oocyte (Leroy et al., 2004).

High energy diets have been shown to lead to elevated levels of total cholesterol in dairy cattle (Varman et al., 1968; Wehrman et al., 1991). Previous studies have evaluated embryo quality and embryo color in relation to circulating cholesterol and triglyceride concentrations of different cattle breeds (Leroy et al., 2004a, 2004b; 2005). Leroy et al. (2005) have found that embryos collected from lactating Holstein are darker than embryos from lactating Belgian Blue females. The same pattern was found when lactating Holstein cows were compared with nonlactating Holstein heifers (Leroy et al., 2005). Embryos that were darker in appearance were reported to have an excessive accumulation of lipid droplets, which may be influenced by the biochemical composition of the embryo (Abe et al., 1999; 2002).

Leroy et al. (2004) found that when follicles grew from small to large size, cholesterol concentrations increased but triglyceride concentration decreased (Leroy et al., 2004), which may directly influence the intracellular lipid content present in the oocyte. Leroy et al. (2005) evaluated embryos collected from lactating Holstein cows and nonlactating Belgian Blue females. Embryos that were produced from lactating Holstein cows were of lower quality and darker in color compared with embryos produced from nonlactating Belgian Blue females. At the time of cryopreservation, only 61% embryos from lactating Holstein and 75% embryos from nonlactating Belgian Blue were of morphological quality to be cryopreserved. Total blood cholesterol concentrations were found to be higher in lactating Holstein females than nonlactating Belgian Blue females, but triglyceride was higher in nonlactating Belgian Blue compared with lactating Holstein females. It was proposed that embryo color, as well as serum cholesterol and triglyceride concentrations, were influenced by breed type. The factors that were found to influence total cholesterol and triglyceride concentrations in donor's blood (e.g., breed, milk yield and nutrition) may indirectly influence embryo color and thus, likely embryo lipid content, freezability and subsequent pregnancy rates.

Hill et al. (1988) superovulated Holstein donors that were supplemented with vegetable oil or beef tallow diets 30 days post embryo collection. The embryos were evaluated for color and then cryopreserved to analyze viability based on pregnancy rates. Higher pregnancy rates after frozen-thawed transfer were obtained from morulae that were light in color compared with transferred morulae embryos that were darker in appearance. However, pregnancy rates from dark colored blastocysts resulted in higher pregnancy rates when compared with lighter colored blastocysts of the same day of age. Morulae produced from donors that had high cholesterol serum concentration resulted in a higher pregnancy rates than donors with low circulating cholesterol concentrations. In addition, donors with a

higher circulating cholesterol concentrations also obtained a higher pregnancy rates at the blastocyst stage compared with donors that had low circulating cholesterol concentrations. Levels of cholesterol in donor cattle may have a direct influence in follicular fluid content of lipids, which may determine the color of the embryo produced from the superstimulated female.

Bovine follicular fluid forms the biochemical environment of the oocyte before ovulation (Edwards, 1974; Chang et al., 1976). During the follicle growth phase, metabolic activity, along with the barrier of the follicular wall, significantly changes with growth of the follicle. Leroy et al. (2004) evaluated metabolite and ionic composition of follicular fluid from different size follicles and noted a positive relationship with circulating levels of cholesterol and triglyceride in lactating dairy cattle. Follicular fluid concentrations of glucose, β -hydroxybutyrate (β -OHB) and total cholesterol increased from small to large follicles while potassium, chloride, lactate, urea and triglycerides decreased in the follicular fluid. A significantly positive correlation was found in large follicles with triglycerides, nonesterified fatty acid (NEFA), total cholesterol, chloride, glucose, β -OHB, urea and total proteins for all follicle size classes evaluated. It was concluded that oocytes and granulosa cells from dairy cattle develop and mature in a changing biochemical environment with the growth of the follicle from small to large size, which may alter lipid composition in the oocytes.

DETERMINATION OF LIPID CONTENT IN OOCYTES AND EMBRYOS

Lipid content can be quantified by several methods. Such methods can be used to determine the role of lipid uptake and its metabolism in oocytes and embryos of different species. Understanding lipid metabolism in oocytes and embryos may lead to improvement of cryopreservation, which may lead to higher post-thaw pregnancy rates in porcine and bovine embryos.

Cellular particles from biological fluids and tissues can be separated by density gradients. Most of the density gradient media have been developed since the 1960s (Pertoft, 2000) and have had several advantages in the field of research. A more practical method of establishing a density gradient and indirect measurement of lipid content is by placing stepped layers of sucrose solutions of different concentrations (i.e, densities) in a test tube, with the heaviest layer at the bottom and the lightest layer at the top. The cell fraction to be separated is placed on top of the layer. A particle will sink if the density of the particle is higher than that of the immediate surrounding solution. The particle will continue to sink until a position is reached where the density of the surrounding solution is exactly the same as the density of the particle.

Centrifugation was introduced in the 1852 and it was used to determine the volume of red blood cells (see review by de Duve, 1971). Centrifugation can be applied to help accelerate the process of reaching the quasi-equilibrium point; however, unlike the differential centrifugation technique used during the first stage of cell separation, the length of centrifugation for this second stage is not critical, as long as the system is permitted to come to quasi-equilibrium (de Duve, 1971). Different separation procedures require centrifugation conditions such as centrifugal force, centrifugation time and type of centrifugation rotor (Pertoft, 2000).

Gas chromatography (GC) has been used to evaluate lipid content present in oocytes and embryos. Kim et al. (2001) analyzed lipid content and fatty acid composition by GC of fresh immature and *in vitro* matured bovine oocytes cultured in medium with or without serum, in addition to frozen-thawed immature oocytes. McEvoy et al. (2000) evaluated fatty acid composition of immature bovine, porcine and ovine oocytes by gas chromatography. Additionally, Ryuichi et al. (1999) used capillary column gas chromatography to evaluate fatty

acid composition of bovine immature oocytes and 2-cell and blastocyst stage embryos cultured in serum-free or serum supplemented medium. The studies demonstrated that gas chromatography is effective in determining fatty acid composition in embryos and oocytes from different mammals. However, between 4 to 100 embryos or oocytes are required to determine lipid content using this approach.

A kit-based assay was developed by Kim et al. (2001) to determine different lipid classes of oocytes and embryos. Triglycerides, total cholesterol, phospholipid (phosphocholine-containing phospholipid) and nonesterified fatty acid content of immature and *in vitro* matured bovine oocytes. With a lipid kit-based assays, a pool of 100 oocytes or embryos are used to determine different classes of lipids.

Enzymatic assay coupled to microscopy detection have been used to measure triglycerides content using 1 to 3 oocytes or embryos. Ferguson et al. (1999) and Sturme et al. (2003) evaluated triglyceride content in porcine and bovine oocytes and embryos using fluorescence microscopy. Using fluorescence microscopy to determine triglyceride content present in oocytes and embryos is sensitive; however, the procedures are difficult to perform.

Ultrastructural composition of lipids in porcine and bovine oocytes and embryos can be evaluated using lipid specific stains. Crosier et al. (2000), Tominaga et al. (2000); Crosier et al. (2001) evaluated ultrastructural morphometry of lipid droplets in bovine zygotes, morulae and blastocysts produced *in vivo* and *in vitro* using transmission electron microscopy after the embryos were stained with Sudan Black B. Kikuchi et al. (2002) evaluated morphological features of lipid droplet transition of porcine oocytes during fertilization and early embryonic development to blastocyst *in vivo* and *in vitro* using Toluidine Blue stain and transmission electron microscopy (TEM). The combination of certain stains and electron microscopy can determine density and size of intracellular lipid droplets in

embryos. However, these techniques do not permit analysis of the whole oocyte or embryo because some slices from the oocyte or embryo are lost during the procedure.

Recently, a lipid specific fluorescent dye has been used to evaluate lipid droplets and lipid content in porcine, bovine and murine oocytes and embryos (Genicot et al., 2005). Nile Red stain can be used to evaluate lipid content on a single embryo rather than sectioning or using 4 to 100 oocytes or embryos. Previously, Nile Red has been used to determine lipid storage in copepods. Carman et al. (1991) used Nile Red to detect lipid droplets inside the copepod oocyte. Bright yellow to orange fluorescence was clearly visible under epifluorescent illumination when copepod oocytes were evaluated. Additionally, Nile Red was used to evaluate the uptake of fatty acid into the copepod oocyte. A detectable decrease in lipid reserve due to starvation was noticed when copepod oocytes were stained. With Nile Red, a decrease in fluorescence was a consequence of consumption of lipid reserves in the oocytes. Additionally, utilization of Nile Red was compared with quick-frozen vs. formaldehyde-preserved copepod oocytes to see if oocytes could be preserved before evaluation with Nile Red stain. Oocytes that were fixed in formaldehyde and stained briefly with Nile Red, showed a detection of intracellular lipid droplets. Additionally, when samples were quick-frozen in liquid nitrogen, and stained with Nile Red, lipid droplets were readily detectable, even if samples were frozen for 3 weeks at -50°C. The amount of intracellular lipids did not differ from that of freshly collected oocytes when compared with frozen oocytes (Carman et al., 1991). Nile Red is sensitive enough to detect lipid droplets in individual benthic copepod oocytes when oocytes were collected fresh, frozen or fixed.

Genicot et al. (2005) used Nile Red to evaluate lipid content of single bovine, porcine and murine oocytes. The peak fluorescence observed corresponds to neutral lipids, which are mainly triglycerides. The emitted fluorescence resulting when oocytes are stained with

Nile Red is specific for lipid droplets (Genicot et al., 2005). No fluorescence is observed in the cytosol or in the nuclear compartment of the oocytes. Staining of oocytes takes 2 hours or longer without significant effect in fluorescence intensity. Genicot et al. (2005) determined lipid content is higher in porcine oocytes when compared with oocytes of cattle and sheep. Such findings support McEvoy et al. (2000) study, that showed that porcine oocytes were higher in lipid content when compared to bovine and ovine oocytes. The lipid content of porcine oocytes was ~2.4 greater than the amount noted in bovine oocytes. Of the 3 species analyzed in the study, ovine oocytes contained fewer lipid content than those of pig and cattle.

Leroy et al. (2005) established a correlation between oocyte and embryo color with fluorescence intensity after staining with Nile Red stain. Darker oocytes and embryos emitted a higher amount of fluorescence when compared with granulated and pale oocytes and embryos. The study also demonstrated that morulas cultured in medium supplemented with serum also emitted a higher amount of fluorescence than embryos cultured in serum-free medium. In general, Nile Red stain is sensitive enough to detect intracellular lipids in oocytes and embryos with quick and accurate results.

MODELS OF LIPID METABOLISM IN OOCYTES AND EMBRYOS

Due to the difficulty of collecting mammalian oocytes and embryos, insect eggs have been used to study fatty acid metabolism in farm and domestic animals. Insect eggs, like ova of other animals, must contain all substances necessary for independent development of the embryo. For proper development, the mature insect oocytes have to contain large amounts of proteins and lipids that serve as building blocks and a source of energy. Lipids, mostly triglyceride and smaller amounts of phospholipid (PL) and cholesterol, make up 30% to 40% of the dry weight of insect oocytes (Ziegler et al., 2006). These lipids may originate

directly from the meal or from storage depots in the fat body. However, accumulation of lipids into oocytes from the body has not been reported.

Mosquitoes are dependent on lipids in their fat body to properly develop future offspring (Ziegler et al., 1997). The primary function of lipids in the fat body is a source of energy. Lipids that are present in the mosquito eggs have been reported to be accumulated from the fat body of the mosquito into the eggs (Ziegler et al., 1997 and Ziegler et al., 2006). The lipid reserves are said to be carried over into the developing larvae for proper development of the oocyte. Van Handel (1993) showed that in the mosquito (*Culex quinquefasciatus*), approximately 90% of the energy used by the developing embryo originates from lipids. In order for female mosquitoes to reproduce, they must ingest protein meals and vertebrate blood provides the exact amount of protein required for oogenesis (Briegel et al., 2002). Blood meals initiate a new gonotrophic cycle, which produces a new single batch of eggs.

Ziegler (1997) studied lipid synthesis in ovaries and fat body of yellow fever mosquito (*Aedes aegypti*). Analysis of the lipid composition by gas chromatography showed that ovaries synthesized mainly phospholipids while mature oocytes have an abundant content of triacylglycerol. These findings indicate that only a small amount of lipids found in the oocytes are synthesized by the ovary. In addition, the ovaries of mosquito mainly synthesize mostly saturated fatty acids while the oocytes contain mostly unsaturated fatty acids (Briegel et al., 2002). Eggs of *Aedes aegypti* cultured *in vitro* contained 80% saturated lipids while 70% were classified as unsaturated in *Aedes aegypti* eggs cultured in maturation medium for 2 days, incorporated 20 µg of intracellular lipids. The lipids incorporated into the *Aedes aegypti* eggs proves that intracellular lipids are synthesized by the female body.

Ziegler et al. (2001) studied the formation of lipid reserves in the fat body and eggs of yellow fever mosquito, *Aedes aegypti*. In mosquitoes that were fed with sugar water, lipids in the ovaries significantly increased over a 7 day feeding. On day 5, mosquitoes that were fed a blood meal and with free access to sugar water also increased lipids in their ovaries. Additionally, fat body lipid content in the mosquitoes gradually decreased over the feeding trial, indicating that lipids were being transferred to the eggs. However, when mosquitoes were starved for 7 days, fat body lipid levels decreased below minimum value with no mature eggs being produced. Once the starved mosquitoes were fed with a blood meal, fat body lipid levels increased, which caused the mosquitoes to produce mature eggs. As reported by Ziegler et al. (2001) the lipids synthesized in the ovaries contribute only a minor portion of the lipids in the eggs and that most lipids acquired by the egg are taken up from extra ovarian sources from within the female body.

Ziegler et al. (2001) did a further investigation of lipids in eggs of *Aedes aegypti*. The female mosquitoes were kept for 4 days with free access to sugar water containing radioactive glucose to evaluate lipid movement from the female body to the developing oocytes. These females were then given free access to non radioactive sugar water for an additional 2 days to reduce the level of radioactivity of total hemolymph. Females were then allowed to take a blood meal from an anesthetized rabbit, and afterwards they were kept for another 2 days with free access to radioactive-free water. Lipids present in the mature oocytes, as well as the body, contained high levels of radioactivity. Briegel et al. (2002) stated that large females transferred most of their pre-blood meal lipid into their ovaries while small females transferred a considerably smaller amount of lipid, but with constant segment of sugar-derived lipids to the ovaries. In both the large and small female mosquitoes, lipid content per oocyte was constant throughout all the cycles. This indicated that most of the

lipids in the oocytes are derived from the fat body, which is the principle storage site of lipids, and are synthesized from carbohydrates ingested before a blood meal.

LIPID POLARIZATION OF BOVINE EMBRYOS

Numerous calves have been born through the use of frozen-thawed *in vivo*-produced embryos (reviewed by Seidel, 2006). However, the demand for *in vitro*-produced (IVP) embryos is increasing due to the rising price of frozen semen and the limited ability to flush donor cattle (Looney, unpublished data). IVP embryos are produced by repeated transvaginal oocyte aspiration or from slaughterhouse ovaries; then matured, fertilized and cultured *in vitro* up to the blastocyst stage. From the production point of view, embryos need to be frozen so transfer can take place at a later date.

In vitro-produced bovine embryos are more sensitive to freezing than *in vivo*-derived embryos (Leibo et al., 1993; Pollard et al., 1993), especially prior to blastocyst stage. Sensitivity of embryos under chilling conditions depends on their developmental stage and culture conditions, serum or serum-free (Leibo et al., 1995), as well as the presence of intracellular lipid droplets in the *in vitro*-produced embryos prior to the blastocyst stage (Plante et al., 1994). Bovine embryos have been shown to contain a large number of lipid droplets at the blastocyst stage, which is coincident with the loss of sensitivity to cooling (Trounson et al., 1976; Mohr et al., 1981).

The increased intracellular lipid content with the relatively smaller inner cell mass may play a crucial role for the *in vitro* embryo and both are probably attributable to the inappropriate culture environment the *in vitro*-produced oocytes and embryos are placed under (Iwasaki et al., 1990; Greve et al., 1993; Leibo et al., 1993). A large amount of intracellular lipid is thought to compromise embryo quality through impaired mitochondrial

function, which can reduce cryotolerance. The reduced cryotolerance results in low pregnancy rates obtained in pig and cattle (Abe et al., 2002).

Nagashima et al. (1994) demonstrated the freezing tolerance of porcine embryos to freezing, 2-cell to 4-cell and 4-cell to 8-cell stages, can be increased by removing cytoplasmic lipid droplets by performing micromanipulation after centrifugation. The removal of lipids from the porcine embryos has produced live birth from thawed cryopreserved embryos. With centrifugation displacing intracellular lipids to one side of the embryo prior to micromanipulation has improved freezability of porcine embryos and IVP bovine embryos at pre-morula stages, regardless of whether they are of *in vivo* or *in vitro* origin (Ushijima et al., 1999).

Murakami et al. (1998) studied the effects of centrifugation and lipid removal on cryopreservation of *in vitro*-produced bovine embryos at the 8-cell stage. The survival and development rate of the delipidated embryos to the blastocyst of frozen-thawed 8-cell embryos increased slightly with increasing force (g) of centrifugation. Development rates were higher in the blastocyst of frozen-thawed 8-cell embryos with centrifugation force at 16,000 x g for 20 minutes. Lipid removal treatments compared to the intact embryo group (control), had a significantly higher development rate. However, the development rate to the expanded-hatched blastocyst stage had no significant difference between groups.

Diez et al. (2001) also investigated the effects of delipidating *in vitro*-produced bovine zygotes and its effect on further development and consequences of freezability. Embryos produced *in vitro* were micromanipulated after high speed centrifugation and then co-cultured with Vero cells with 10% FCS. The embryos in the treatment groups were either delipidated, sham-treated (centrifuged but not delipidated) or untreated (control). The blastocyst rates obtained from the groups were 42.1, 42.3 and 39.9%, respectively. Survival rates from

embryos that were frozen/thawed then co-culture for 72 hours with Vero cells with 10% FCS indicated that delipidated embryos had a significantly higher hatching rate after being cultured for 3 days compared with embryos that were not delipidated and cultured. Vajta et al. (2004) had similar results when bovine blastocyst embryos were delipiated and frozen-thawed. Delipidated embryos had a significantly higher hatching rate when compared to non-delipidated embryos. The delipidation of 2-cell stage bovine embryos was comparable with the control *in vivo*-derived embryos. Thus, delipidation of porcine and bovine embryos has a beneficial effect on their tolerance to cryopreservation at the blastocyst stage (Nagashima et al., 2004).

Tominaga et al. (2000) evaluated bovine embryos at the zygote to 8-cell stage to determine the effect of polarization of lipid droplets in the cytoplasm of *in vitro*-produced bovine embryos by centrifugation at 15,500 x g with or without micromanipulation. Polarized bovine embryos were cultured to the 16-cell stage and classified as either mostly or partially delipidated by the degree of lipid droplet removal and then cryopreserved to evaluate post-thaw development. Bovine embryos centrifuged at the 2-cell stage developed to the 16-cell stage similarly to 8-cell stage that were centrifuged. Development rates of frozen-thawed 16-cell stage bovine embryos to the blastocyst were significantly higher when delipidation was performed at the 2-cell rather than at the zygote stage. The displacement of intracellular lipids droplets at the 2-cell stage by centrifugation and micromanipulation improved the survival rate of primarily delipidated 16-cell embryos after cryopreservation.

DELIPATION OF PIG EMBRYOS

Porcine oocytes and embryos from early cleavage to blastocyst stage are known to be highly sensitive to cryopreservation temperatures below 15°C (Wilmut et al., 1972; Polge et al., 1974), which limits their ability to be cryopreserved with conventional methods. Live

births of piglets from cryopreserved embryos are low due to their structural sensitivity to cryopreservation (Hayahi et al., 1989; Kashiwazaki et al., 1991). The rate of cooling to critical temperatures has been shown to affect bovine embryo survival (Wilmut, 1986). Low pregnancy rates are common in porcine embryos that are cooled to -15° or -20°C (Dobrinsky, 2002). Conventional freezing survival rate has been reported to be ~30% in porcine embryos (Nagashima et al., 1994). The restriction of cryopreserving porcine embryos has limited the use of transferring frozen embryos in the commercial industry and severely limits the practical application of this technology to improve genetics.

Research has extensively focused on improving the cyropreservation procedures of porcine embryos. Various reports have suggested that high intracellular lipid content found in the embryos are linked to the low survival rate of chilled or cryopreserved porcine embryos (Nagashima et al., 1994). Porcine oocytes and early embryos appear darker, which is associated with a high quantity of endogenous lipids (Sturmey et al., 2003). Porcine embryos contain a large number of lipid droplets and are abundant at the 2-cell to 8-cell stage and decline markedly at the blastocyst stage (Niimura et al., 1980). Porcine embryos at the perihatching stage are known to be less sensitive to low temperatures and their cyropreservation by conventional freezing method has successfully led to offspring production (Nagashima et al., 1989; Nagashima et al., 1992).

Research suggests that the embryonic cytoskeleton is susceptible to damage during cyropreservation, and this cellular disruption may be averted by using cytoskeleton stabilizers before preservation (see review by Dobrinsky, 1997). Porcine embryos cryopreserved by conventional freezing and vitrification under the influence of cytoskeletal stabilization have resulted in pregnancies of live offspring from recipient females after surgical transfer (Dobrinsky, 1997). However, the cryo-tolerance of porcine embryos at early

cleavage stages may also be increased after decreasing their relatively high content of intracellular lipid droplets.

Nagashima et al. (1994) proposed that the removal of cytoplasmic lipids may enhance the cryo-tolerance of porcine embryos to chilling. Cryopreservation of porcine embryos has been improved by isolating the lipids from the embryo by high speed centrifugation, and then micromanipulated to remove the lipids before being cryopreserved (Nagashima et al., 1994). Although, the technique is timely, live births have resulted from thawing of porcine embryos where the cytoplasmic lipids were removed. Delipidated fertilized zygotes that were chilled, cleaved at the 2-cell and 4-cell stages and continued to develop beyond the 8-cell stage, while non delipated embryos (controls) lysed within 24 hours post-chilling. Porcine embryos gain tolerance when the intracellular lipid content in the cytoplasm is removed by micromanipulation, which indicates that high sensitivity of porcine embryos to chilling is related to their lipid content (Nagashima et al., 1994; Dobrinsky et al., 2001). Pregnancy from porcine embryos cryopreserved can be improved by reducing the amount of lipid content present in the embryo (Ritsuko et al., 2004).

Mature porcine oocytes have not been effectively cryopreserved due to their high lipid content and structural changes after cooling and freezing (Arav et al., 1996). Park et al. (2005) showed that partial removal of cytoplasmic lipid on the survival of vitrified germinal vesicle (GV) stage porcine oocytes can be improved by micromanipulation. Porcine oocytes were either delipidated, just centrifuged with no delipation or left as control. Oocytes that were cultured after centrifugation had fewer oocytes that reached metaphase-II (M-II) compared with non centrifuged oocytes. Once oocytes in the centrifuged group reached M-II stage, no further reduction in the M-II rate was detected. Porcine oocytes that were cryopreserved by vitrification and then re-warmed showed that oocytes in the delipated group

reached the M-II stage in culture compared with non delipated oocytes. From the results, it shows that the removal of lipids from GV stage pig oocyte by micromanipulation and then cryopreserved by vitrification can enhance *in vitro* maturation.

VITRIFICATION OF PIG EMBRYOS

Vitrification is a relatively new approach of cryopreservation that achieves rapid freezing without the use of a freezing machine (Niemann, 1991). Presently, vitrification is regarded as an alternative to traditional slow freezing procedures, which results in satisfactory cryopreservation of porcine embryos (Vajta, 2000). Live births of piglets after transfer have resulted from the development of vitrification system after pre-treated (delipidated) or untreated vitrified-thawed porcine embryos (Cuello et al., 2004). Recently, Pryor et al. (2007) demonstrated that pregnancy rates from frozen Brahman *in vivo* derived embryos can be improved by vitrification when compared with conventional direct transfer. Before vitrification can be applied into conventional cryopreservation, research is needed to understand the factors that affect embryo survival after vitrification and the technical aspects of the procedures.

Esaki et al. (2004) studied vitrification of porcine embryos derived from *in vitro*-matured oocytes. Porcine embryos were cryopreserved by vitrification using the minimum volume cooling (MVC) method and delipation by micromanipulation was performed by polarizing the lipids in the cytoplasm with centrifugation. The delipation group was compared with porcine embryos that were left intact and then cryopreserved by the same MVC method. Delipated and vitrified embryos survived (36% survival rate) 24 and 48 hours after thawing. Most of the embryos in the treatment groups of delipation hatched during culture but had a low survival rate (9.7% survival rate). Vitrification and delipation of lipids in porcine embryos provides an effective way to cryopreserve porcine embryos with success after transfer.

Cuello et al. (2004) evaluated vitrification of porcine embryos at various developmental stages using different ultra-rapid cooling procedures. Different vitrification systems were compared to evaluate the cooling rate on *in vitro* development of vitrified-thawed porcine morulae, early blastocysts or expanded blastocysts produced *in vitro*. Open pulled straw (OPS), superfine open pulled straws (SOPS) and Vit-Master-SOPS were the 3 vitrification procedures compared. The development stages of the porcine embryos at collection affected the survival and hatching rate of vitrified-thawed embryos. Embryos vitrified at the expanded blastocyst stage showed survival and hatching rates similar to those of fresh expanded blastocysts. Hatching rates from vitrified porcine morulae or early blastocysts had less development when compared with fresh blastocysts. Raising the cooling rate above 20 °C/minute during vitrification does not enhance *in vitro* development of porcine embryos at the morula, early or expanded blastocyst stages after warming (Cuello et al., 2004). However, vitrification of porcine embryos was effective for preserving early or expanded blastocysts.

EFFECT OF SERUM IN OOCYTES AND EMBRYO LIPID CONTENT

Bovine embryos produced by IVM and IVF are routinely cultured in serum-supplemented medium with or without somatic cell co-cultures. *In vitro* culture conditions are fundamentally different from *in vivo* environment (Lonergan et al., 2003). Serum content in the medium is beneficial for embryonic development due to the presence of growth factors and chelators of heavy metals. However, the addition of serum to culture media may contribute to the large offspring syndrome and accumulation of cytoplasmic lipids in the developing embryo (Looney, unpublished data). *In vitro*-produced bovine embryos in the presence of serum have been successfully cryopreserved at the expanded blastocyst stage (7 days after insemination), with hatching rates of 50% to 80% (Semple et al., 1995).

Standard cryopreservation methods yield higher survival rates for *in vivo*-produced embryos compared with *in vitro*-derived embryos that yield significantly lower survival rates (Greve et al., 1993; Leibo et al., 1993). Research has focused on serum-free culture media to determine if absence of serum can improve the survival rate after cryopreservation.

Abe et al. (2002, 2003) evaluated bovine embryos developed from *in vitro*-matured (IVM) and fertilized (IVF) oocytes cultured in either serum-free or serum-containing medium (HPM 199 + 5% FCS). Bovine embryos that were cultured were stained with Sudan Black to evaluate intracellular lipid content in the embryo. Embryos in serum-supplemented medium emitted a higher fluorescence, which indicated higher intracellular lipid droplets compared with those cultured in serum-free medium. Additionally, cytoplasmic lipid droplets (LD) of bovine embryos from 2-cell to hatched blastocyst stages, evaluated by electron microscopy, were more abundant in embryos cultured in serum-supplemented medium. Additionally, the post-thaw survival and hatching rate of morulae from serum-containing medium was lower compared with serum-free medium. The addition of serum in culture medium increased lipid content in embryos during development, which may be associated with the incorporation of lipoproteins from the serum, and may result in impaired function of mitochondria (Abe et al., 2003). Moreover, the increased accumulation of cytoplasmic lipids in the embryos has a negative effect on cryopreservation of bovine embryos (Pollard et al., 1994).

Semple et al. (1995) evaluated cryopreservation of *in vitro*-derived bovine embryos produced in a serum-free culture system. Bovine zygotes were subsequently co-cultured with bovine oviductal epithelial cell (BOEC) explants in either unsupplemented, serum-free (Menezes B2) medium or B2 medium supplemented with 10% steer serum. Frozen-thawed blastocysts (day 7) were cultured for 72 hours in B2 medium to evaluate further development. Developing bovine embryos had that serum-free cleavage rates were higher

compared with embryos in medium with serum added. The survival rates between the groups (re-expansion within 24 hours) were higher in the serum-free than embryos in the serum added medium. However, hatching rates from embryos in the serum-free compared with embryos in serum medium were similar. Results indicate that serum in medium is not essential at any time after fertilization to produce embryos with the ability of undergoing cryopreservation. With the removal of serum (BSA) in medium, lipids may influence the stage-dependent sensitivity to cryopreservation of *in vitro*-produced bovine embryos (Semple et al., 1995).

CHAPTER III

EVALUATION OF INTRACELLULAR LIPID CONTENT USING SUCROSE BUOYANT DENSITY GRADIENT AND NILE RED STAINING IN MATURE OOCYTES

INTRODUCTION

Oocytes from most mammalian species are sensitive to low temperatures (Leibo et al., 1995). In some species, the poor survival rate after thawing has been linked to the presence of high intracellular lipid content present in the oocyte (Young et al., 1994). Generally, mammals contain numerous endogenous lipids in their oocytes.

Few reports have investigated lipid content of mouse oocytes and embryos due to their relatively small size and low abundance of lipids (Hishinuma et al., 1985). Loewenstein and Cohen (1964) reported that 12.5% of the cytoplasmic dry mass of 2-cell mouse embryos contained lipids. Hishinuma et al. (1985) found large and medium lipid droplets to be abundant in morula and blastocyst stage mouse embryos.

McEvoy et al. (2000) determined that pig oocytes have 161 ± 18 μg of fatty acid mass, which is ~2.5-fold greater than the amount of fatty acids found in cattle and sheep oocytes (63 ± 6 μg and 89 ± 7 μg , respectively). Homa et al. (1986) and Sturmey et al. (2003) also concluded that pig oocytes and embryos have a larger quantity of endogenous lipid compared with beef cattle and sheep. It has also been established that there is no significant difference in lipid content between beef cattle and sheep (McEvoy et al., 2000). The low survival rate after cryopreservation of porcine embryos (Nagashima et al., 1994; Leibo et al., 1995) could be attributed to the high lipid content present in porcine embryos.

Utilization of a buoyant density gradient has traditionally been applied to determine the mass density of embryos (Knutsen et al., 2001); however, Nile Red staining allows for a more precise quantification of lipids (Crosier et al., 2000, Tominaga et al., 2000, Crosier et

al., 2001; Kikuchi et al., 2002). Nile Red is the most common stain used for the specific detection of intracellular lipid droplets in oocytes and embryos (Genicot et al., 2005). Genicot et al. (2005) indicated that porcine oocytes contained ~2.4-fold more lipid droplets compared with bovine oocytes when evaluated with Nile Red fluorescence intensity. Murine oocytes contained fewer lipids when compared with porcine or bovine oocytes.

The objectives of this study were: (1) To compare lipid content in murine, porcine and bovine oocytes using a sucrose step buoyant density gradient and Nile Red staining and (2) to standardize a protocol for the quantification of intracellular lipid content in oocytes using buoyant density and Nile Red staining.

MATERIALS AND METHODS

Experimental Design

In Experiment 1, lipid content was determined in (A) murine (n=50), (B) porcine (n=50) and (C) bovine (n=50) mature metaphase II (M-II) oocytes using a sucrose buoyant density gradient. Three replicates were performed within a 3 month period.

In Experiment 2, *in vitro*-matured bovine oocytes were used to determine the optimal incubation time in Nile Red to quantify lipid content. M-II (n=15) oocytes were incubated with Nile Red solution for 2, 4, 8, 12, 20 and 24 hours. Scion Image (Scion Corp., Frederick, MD) was used to calculate the mean of Nile Red Units (NRU) (high NRU = higher lipid content while low NRU = lower lipid content) in oocytes.

In Experiment 3, mature oocytes from murine, porcine and bovine (n=50) were evaluated for lipid content by staining with Nile Red for 24 hours.

Experimental Procedures

Experiment 3.1

Oocyte Collection

Laboratory mice (*Mus musculus*) were stimulated by administering 5 IU of Equine Chorionic Gonadotropin (eCG) via intra peritoneal (IP) injection. At 46 hours post- eCG, mice were administered 5 IU of Human Chorionic Gonadotropin (hCG). Females were mated with vasectomized males after the administration of hCG to induce ovulation. Mice were checked for a vaginal plug the following morning after exposure to the males. A distinct vaginal plug indicated a mating with the vasectomized male. M-II oocytes were dispensed into a 1.5 ml plastic Eppendorf flex tube (VWR International, West Chester, PA) containing Dulbecco's PBS. Tubes containing oocytes were placed in a sealed box and transported to the Embryo Biotechnology Laboratory (EBL) (Louisiana State University Agriculture Center, Baton Rouge, Louisiana). Upon arrival at the laboratory, tubes were gently swirled and the oocytes were placed into a 30 x 10 mm disposable sterile plastic petri dish (Falcon[®], Beckon Dickinson and Company, Franklin Lakes, NJ). Oocytes with an extruded polar body were visualized using a Nikon SMZ-2B stereoscope (Nikon, Inc., Melville, NY) and were recovered using a glass pipette.

Ovaries from crossbred sows and cows were obtained from local abattoirs (Roucher's Market, Plaquemine, LA; Hydes, Robert, LA, respectively). Ovaries of different species were harvested from mature females of various weights and reproductive stages (Appendix K). Porcine and bovine ovaries were transported in 0.9% saline solution in a sealed container at room temperature. Upon arrival at the laboratory, ovaries were cleaned with ethanol-soaked gauze pads (4 x 4, Johnson and Johnson[™], Arlington, TX) to remove any excess debris and blood.

Cumulus-oocyte complexes (COCs) were aspirated from porcine and bovine ovaries (Appendix A). Antral follicles (2 to 5 mm and 3 to 10 mm for pig and cow, respectively) were aspirated with a 20-gauge, 3.81 cm needle (Monoject, Sherwood Service AG, Mansfield, MA)

attached to a 10 ml air tight syringe (Norm-Ject, Henke-Sass, Wolf GmbH, Tuttlingen, Germany). Follicular fluid containing COCs were dispensed into a 50 ml sterile plastic centrifuge tube previously filled with 25 ml of oocyte collection medium (OCM) (Appendix B and C) maintained at 38.5°C. After all follicles were aspirated and dispensed into the tube, 5 minutes were allowed for oocytes to settle to the bottom of the 50 ml tube. The COCs were collected from the tube using a 1 ml sterile disposable serological pipette (Corning®, Corning, NY) attached to a pipette bulb (VWR International, West Chester, PA). Medium was drawn from the bottom of the tube and dispensed into a 100 x 15 mm integrid™ petri dish (Falcon®, Becton Dickinson, Franklin Lakes, NJ).

In Vitro Maturation

Oocytes with at least 3 layers of cumulus cells were recovered from the search dish and placed in a 30 x 10 mm disposable sterile petri dish (VWR International, West Chester, PA) containing Tissue Culture Medium-199 with Earle's salts (TCM-199; Gibco BRL, Grand Island, NY). Oocytes with intact cumulus cells were washed 4 times in TCM-199.

Bovine COCs were matured in TCM-199 with 10% heat-inactivated characterized fetal bovine serum (Hyclone Laboratories, Logan, UT), 0.7 IU/ml of bFSH, 37.5 IU/ml of bLH and 1 mg/ml of E₂ beta (Sioux Biochemical, Sioux City, IA) (Appendix C). Porcine COCs were matured in TCM-199 with 10% heat-inactivated characterized fetal bovine serum supplemented with 0.5 IU/ml of bFSH and bLH (Appendix B). Bovine and porcine COCs were subsequently grouped by species and matured for 20 and 42 hours, respectively, in 4-well disposable Nunc dishes (Nunclo™ Surface, Roskilde, Denmark) containing 500 µl of culture medium in each well. Dishes were placed in a humidified auto flow water-jacketed incubator at 39°C with 5% CO₂ in air. A group of 48 to 52 porcine and ~25 bovine COCs were cultured in separate wells.

After maturation, cumulus cells were removed using an Analog Vortex Mixer (VWR™ International, West Chester, PA) at a high speed for 3 minutes in 1 ml HEPES buffered with 1 mg of hyaluronidase (obtained from testicular tissue) in a 15 ml plastic conical tube. M-II oocytes with a distinct polar body indicating nuclear maturation, were selected using a Nikon SMZ-2B stereoscope (Nikon, Inc., Melville, NY) and a glass pipette (Figure 3.1). Oocytes were placed in a 4-well petri dish with Dulbecco's phosphate-buffered saline (PBS) before determination of lipid content via sucrose buoyant density gradient.

Buoyant Density Measurement

Fresh sucrose medium consisting of Dulbecco's PBS and the appropriate amount of sucrose was prepared in sterile 50 ml centrifuge tubes. A 500 µl volume of each sucrose level was added to a 5 ml culture test tube (VWR International, West Chester, PA) using a 1 ml sterile disposable serological pipette (Corning®, Corning, NY) attached to a pipette bulb (VWR International, West Chester, PA). Gradient steps ranged from 14 to 30% sucrose in a 2% increment (Appendix D). Oocytes that settle at 14% will have more lipids compared with those that will settle at 30% sucrose level (Appendix L).

Mature oocytes used for buoyant density measurements were washed 2 times in Dulbecco's PBS followed by 2 washes in Dulbecco's PBS with 14% (top layer) sucrose. Washing oocytes in sucrose before taking the measurements allowed them to better settle through the gradient. This step is necessary to prevent oocytes from floating to the top and sticking to the meniscuses. The oocytes were removed from the sucrose wash using a glass pipette and gently dispensed into the top layer of the gradient. A total of 10 oocytes per tube were measured at a time. Oocytes were observed as they settled through the gradient by using a Nikon stereoscope (Nikon Inc., Melville, NY) placed horizontally to evaluate oocyte migration (Appendix M). The oocytes were allowed to settle 1 hour and then were

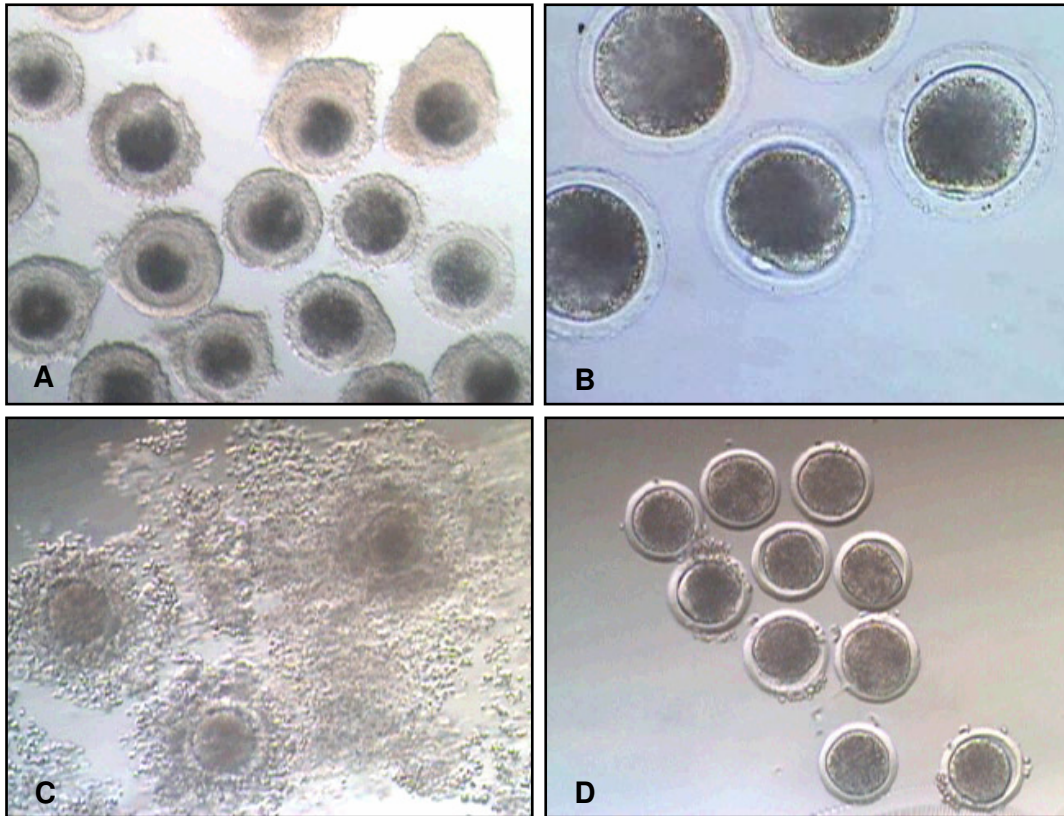


Figure 3.1. A. Porcine cumulus oocyte complexes (COCs) after aspiration. B. Denuded M-II porcine oocytes (20X magnification). C. Bovine (COCs) 20 hours post-maturation. D. Denuded M-II bovine oocytes (A,C and D are at 10X magnification).

individually recovered from their respective level with a glass pipette.

Experiment 3.2

Oocyte Collection

Ovaries from cows were obtained from a local abattoir (Hydes, Robert, LA).

Transportation and COCs retrieval were performed in the same manor as previously described in Experiment 1.

In Vitro Maturation

In vitro maturation of bovine COCs was conducted using the same procedure as previously stated in Experiment 1.

Nile Red Staining

M-II oocytes to be evaluated with Nile Red were incubated in 0.25 ml plastic sterile straws (Agtech, Inc., Manhattan, KS) (1 to 10 oocytes per straw) containing glutaraldehyde/formaldehyde fixative medium (Appendix E). Bovine oocytes were stored in an insulated container on ice at the Louisiana State University Agriculture Center of Reproductive Biology Center (LSU AgCenter RBC, Baton Rouge, LA) for 25 hours before staining. Oocytes were recovered from the straws and placed into 35 x 10 mm disposable sterile petri dishes (Falcon[®], Becton Dickinson and Company, Franklin Lakes, NJ). The samples were placed into sterile 4-well Nunc dishes (Nuncclon, Intermed, Roskilde, Denmark) and washed 4 times with Dulbecco's PBS. Droplets of Nile Red (1 µg/ml) working solution (Appendix F) were prepared in a 35 x 10 mm petri dish. The oocytes were rinsed 5 times in Nile Red stain solution before being placed in the final droplet (30 µl). Oocytes were incubated in Nile Red at room temperature (24°C) inside a sealed cardboard box, so the oocytes would be in total darkness for 2, 4, 8, 12, 20 and 24 hours (Figure 3.2).

Oocytes were recovered and washed 2 times in Dulbecco's PBS after the different

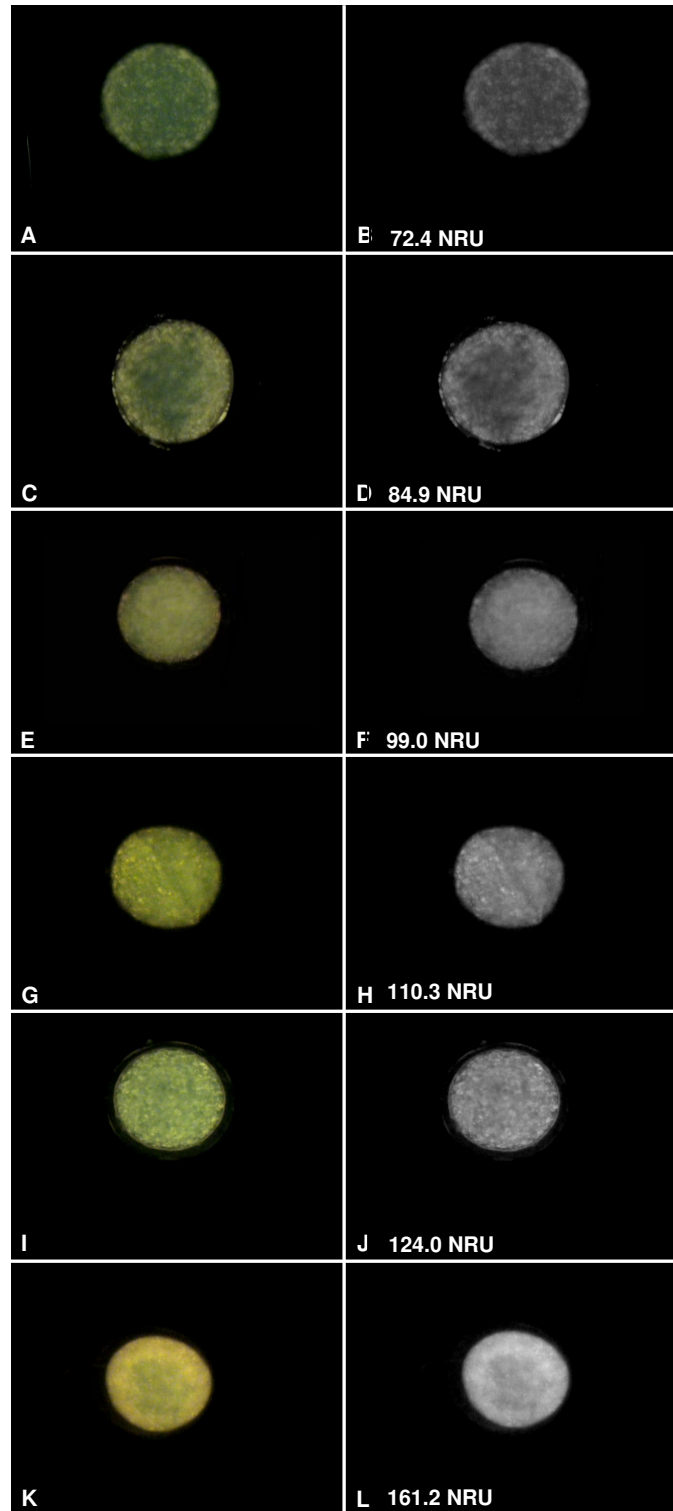


Figure 3.2. M-II stage bovine oocytes stained with Nile Red for different periods of time. Panels A, C, E, G, I and K represent oocytes stained with Nile Red for 2, 4, 6, 8, 12, 20 and 24 hours, respectively. Panels B, D, F, H, J and L are Scion images that measured the fluorescence of each oocyte after 2, 4, 6, 8, 12, 20 and 24 hours of incubation with Nile Red, respectively.

incubation times in Nile Red. Oocytes were then suspended in 10 µl of Invitrogen ProLong[®] Gold antifade reagent (Molecular Probes, Inc., Eugene, OR) on a 25 x 55 mm slide. Three oocytes were placed on each slide. A cover slip was carefully placed over the droplet of ProLong[®] Gold and sealed with nail polish.

Evaluation of oocytes stained with Nile Red was performed by using a Nikon Optiphot microscope (Nikon, Inc., Melville, NY) equipped with epifluorescent illumination and an FITC dichroic filter cube with a long phase barrier filter. Images of each oocyte were captured with phase and fluorescence microscopy, using a Hitachi D20b camera attached to the microscope and stored on a PC computer using Epix software. After images were captured, Scion Image software (Scion Corporation, Frederick, MD) was used to evaluate the mean fluorescence intensity of the Nile Red staining in the oocytes. The program uses an 8 bit gray scale to determine the level of fluorescence.

Experiment 3.3

Oocyte Collection

Ovaries from mature sows and cows were transported and aspirated in the same manner as previously described in Experiments 1 and 2. Murine oocytes were obtained, as described previously in Experiment 1.

Nile Red Staining

Staining of M-II bovine, porcine and murine oocytes with Nile Red was performed using the same procedures as previously stated in Experiment 2, but was incubated for 24 hours (Figure 3.3).

Statistical Analysis

Data for this study were processed using SAS software Version 9.1.3 (SAS Institute Inc., Cary, NC) and analysis was performed using the LS-Means. Differences between

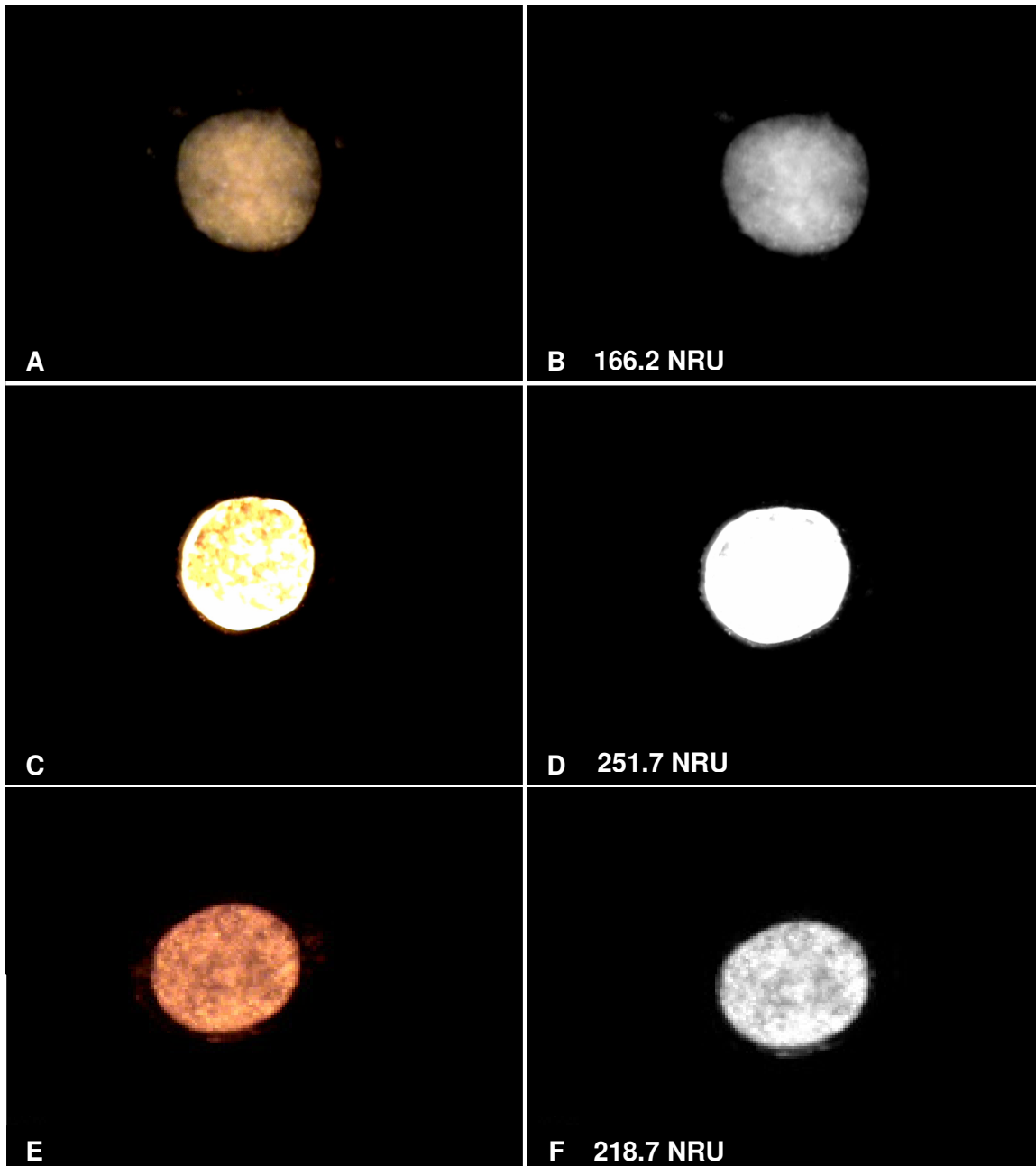


Figure 3.3. Panels of M-II stage murine, porcine and bovine oocytes stained with Nile Red (A, C and E, respectively) and evaluated with Scion Image for NRU (B, D and F, respectively).

treatments were considered significant at the P level of 0.05. The mean of oocyte settling interval in a sucrose buoyant density gradient, the mean fluorescence of oocytes at different times and the mean of fluorescence of oocytes from different species were used as end points for comparison of treatments.

RESULTS

Experiment 3.1

Buoyant Density

Lipid content in M-II porcine oocytes was significantly ($P < 0.001$) lower than in mature bovine oocytes, when calculated using a buoyant density gradient (Figure 3.4). The overall lipid content means in porcine and bovine oocytes for the 3 aspirations were $22\% \pm 0.37$ and $28\% \pm 0.24$, respectively. Intracellular lipid content of mature murine oocytes could not be determined using buoyant density measurement because of their small size and similar refractive index to the sucrose gradient made observations difficult with the stereoscope.

The mean sucrose concentrations of M-II porcine oocytes among the 3 replicates were 23 ± 0.83 , 22 ± 0.80 and $22\% \pm 0.31$. M-II bovine oocytes for the 3 replicates settled at 26 ± 0.00 , 29 ± 0.25 and $30\% \pm 0.06$ in the sucrose gradient. Among porcine and bovine M-II stage oocytes settling in the buoyant density gradient there was significant difference ($P < 0.05$) detected between each aspirations (Figure 3.5).

In conclusion, the buoyant density gradient technique was successfully used to determine differences in lipid content between porcine and bovine M-II oocytes.

Experiment 3.2

Incubation Time of Nile Red

The mean overall fluorescence intensity of M-II bovine oocytes stained with Nile Red for the different staining timed trials of 2, 4, 8, 12, 20 and 24 hours were: 75.64 ± 0.79 ,

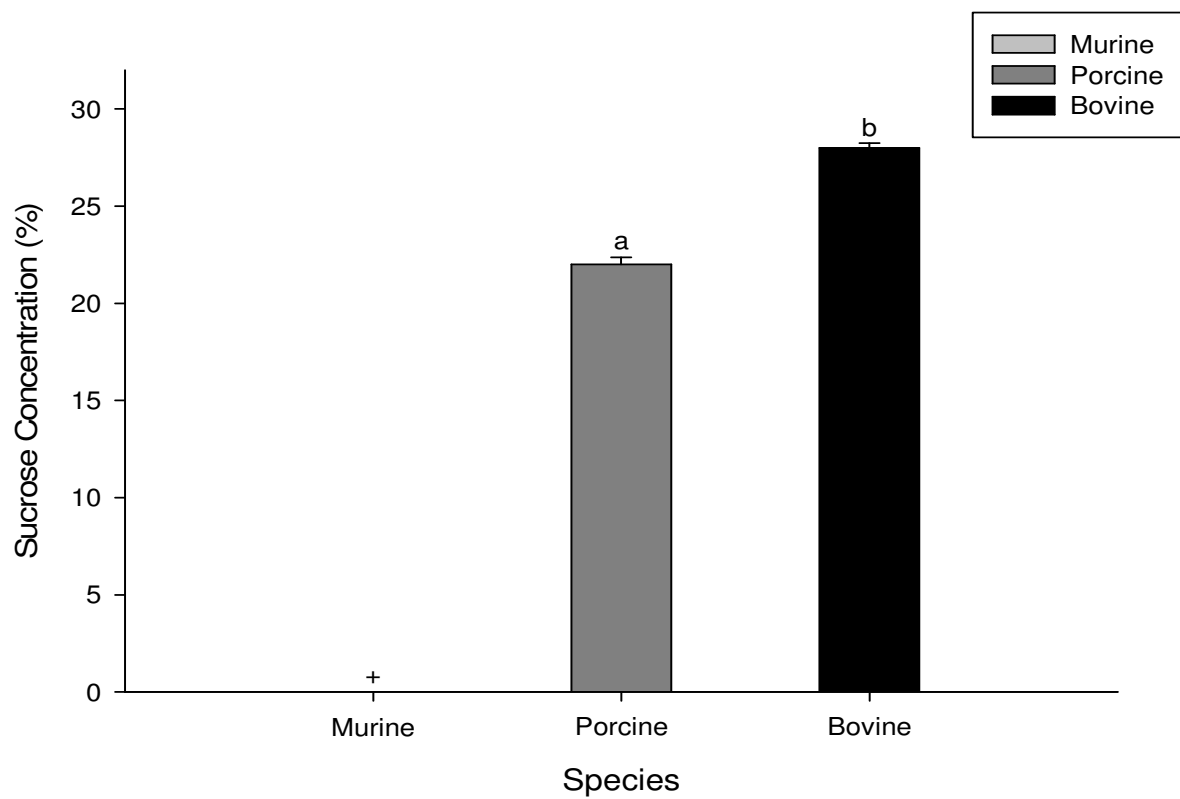


Figure 3.4. Mean (\pm SEM) buoyant density of M-II stage mouse, porcine and bovine oocytes settling in sucrose concentration. ^{a,b}Mean values with different subscripts between columns are significantly different ($P < 0.05$). ⁺Murine oocytes were not evaluated due to the difficulty observing them in the sucrose.

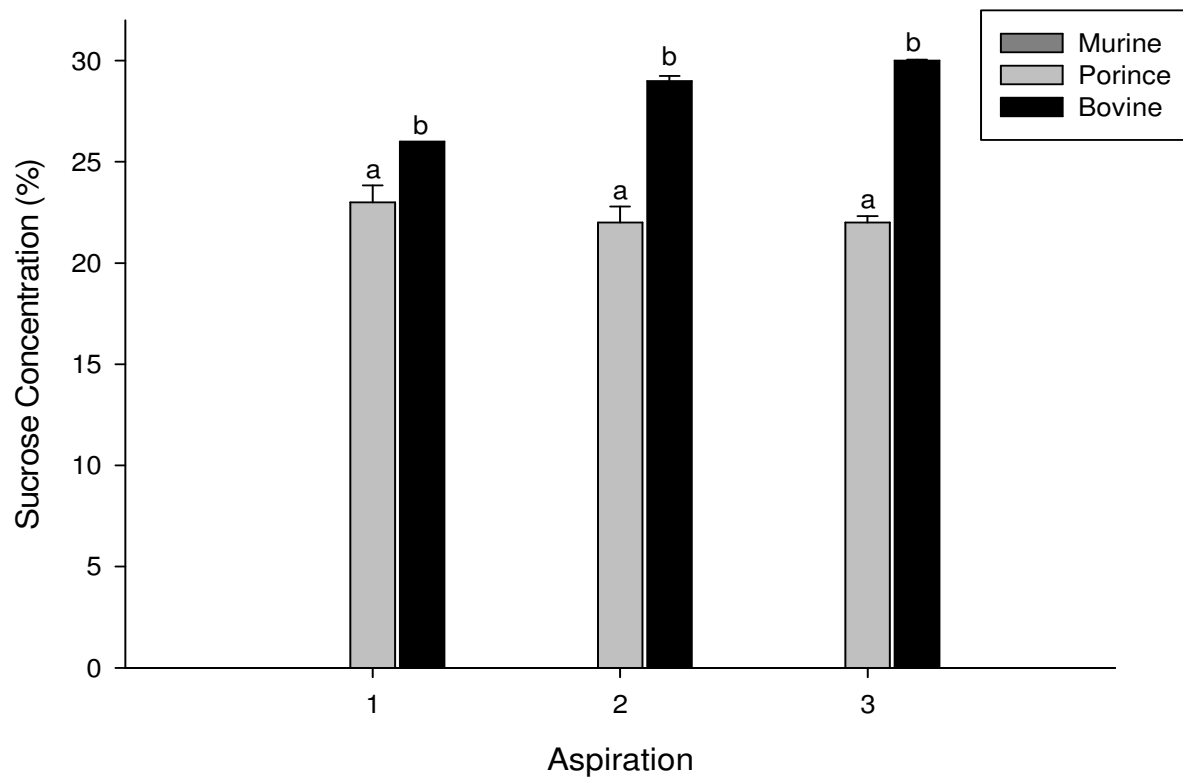


Figure 3.5. Buoyant density concentration (mean \pm SEM) of M-II stage murine, porcine and bovine oocytes settling in sucrose concentration by aspiration. ^{a,b}Mean values with different subscripts within aspiration between species are significantly different (P<0.05).

85.11±5.56, 94.90±0.46, 107.14±0.63, 127.81±1.92 and 154.50±1.59 NRU, respectively (Figure 3.6). The data demonstrated that fluorescence intensity increases with incubation time in Nile Red.

The data showed that 24 hours of incubation in Nile Red produced a higher fluorescence intensity compared with the other incubation times. However, extended time in Nile Red could lead to even higher fluorescence level for a given species.

Experiment 3.3

Lipid Content of Mouse, Porcine and Bovine Oocytes by Nile Red Staining

The mean overall observed fluorescence was significantly higher ($P>0.001$) in porcine compared with murine and bovine oocytes. Additionally, bovine oocytes fluoresced brighter than murine oocytes ($P<0.001$) (Figure 3.7). Furthermore, the mean NRU observed in murine, porcine and bovine oocytes was: 141 ± 1.34 , 246 ± 3.32 and 226 ± 1.31 , respectively. Accordingly, porcine oocytes have the highest level of lipids followed by bovine. Murine oocytes have the lowest lipid content of the three species analyzed in this study.

In conclusion, Nile Red can detect a difference in lipid content between murine, porcine and bovine M-II oocytes.

DISCUSSION

Lipid analysis of oocytes and embryos from mice, pigs and cows has been performed by gas chromatography (Ryuichi et al., 1999, McEvoy et al., 2000; Kim et al., 2001). The use of gas chromatography has been beneficial in determining the amount of lipids found in both oocytes and embryos. These evaluations have been performed to analyze different culture methods and embryos produced *in vivo* compared with *in vitro*. Despite the numerous advantages of gas chromatography, this technique is laborious and requires specialized equipment.

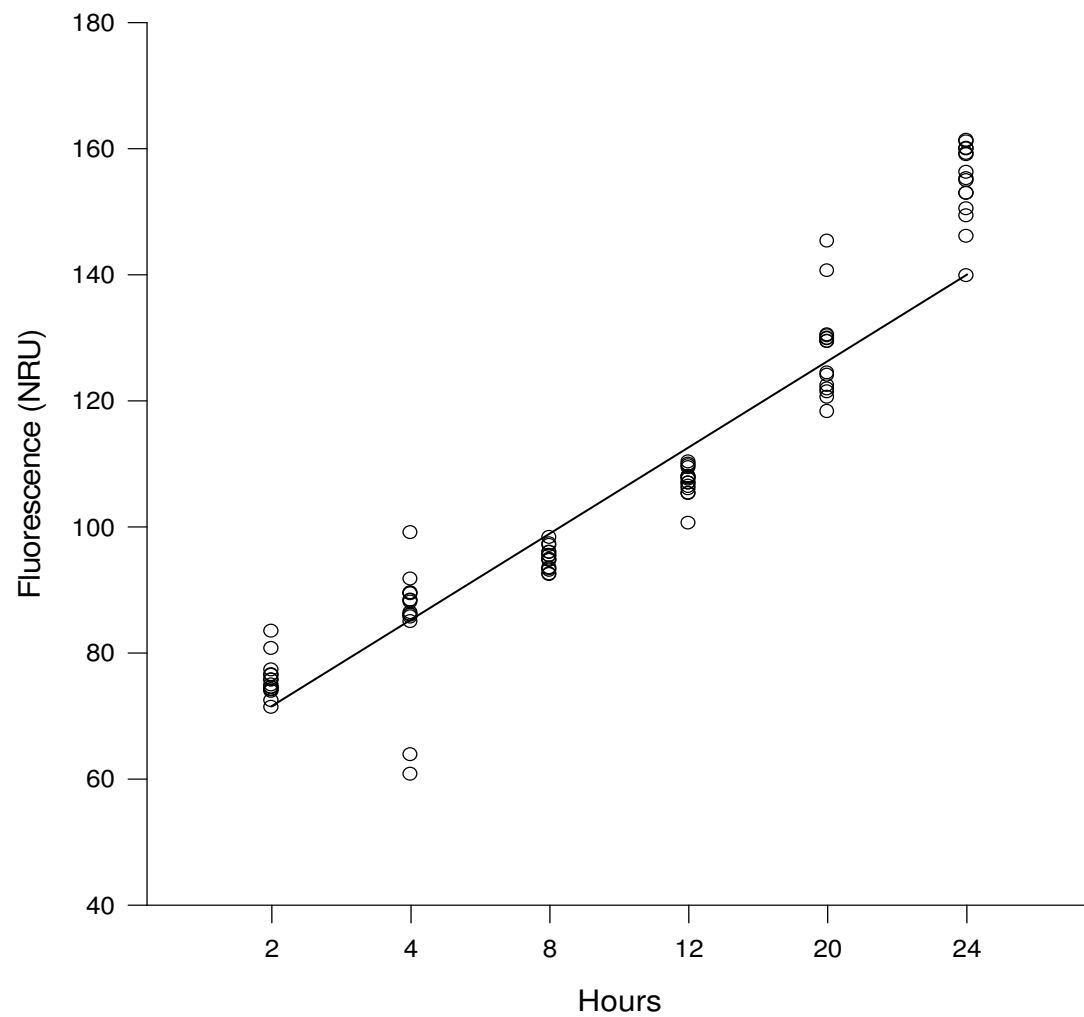


Figure 3.6. Scatter plot of fluorescence intensity (mean \pm SEM) of M-II stage bovine oocytes stained with Nile Red for different period of time. Regression line indicates the best fit with different period of time.

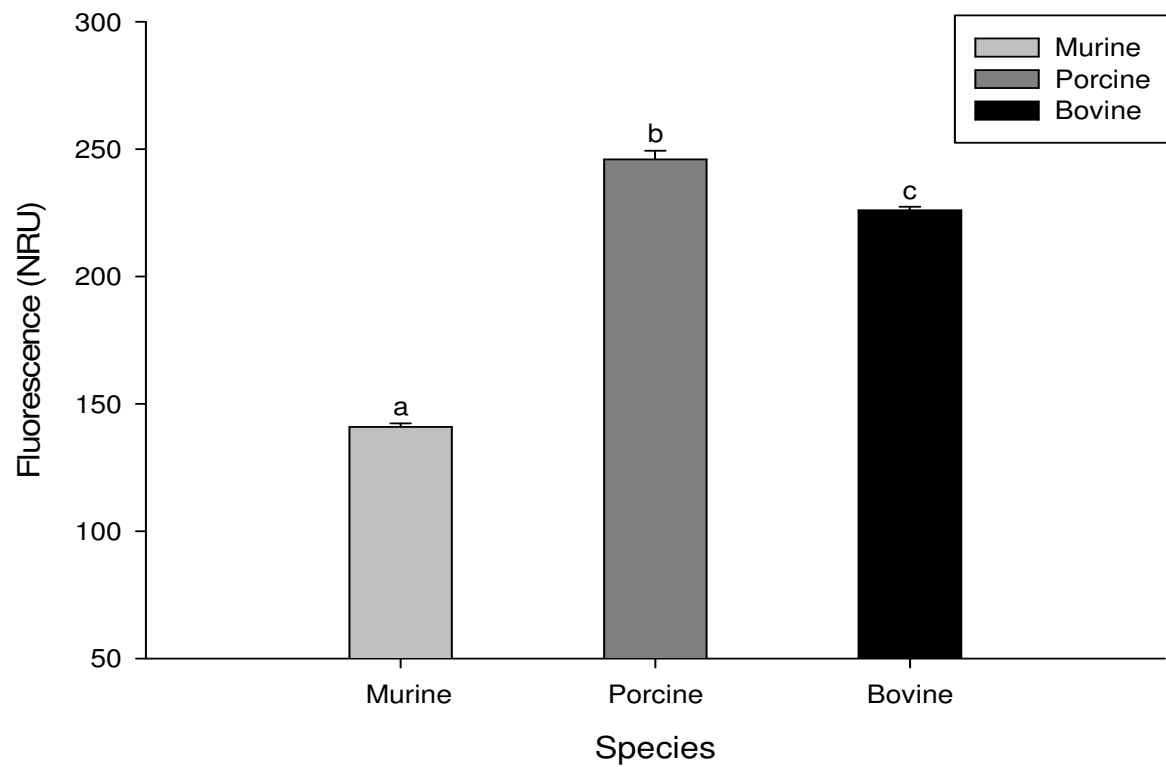


Figure 3.7. Fluorescence intensity (mean±SEM) of M-II stage murine, porcine and bovine oocytes stained with Nile Red. ^{a,b}Mean values with different subscripts between species are significantly different (P<0.05).

Alternatively, the utilization of a buoyant density gradient is an inexpensive and easy technique to perform when only a qualitative estimate of lipid content is needed. In addition, buoyant density measurement has been used to separate cellular particles from biological fluids and tissues. For example, Baltus et al. (1968) isolated deoxyribonucleic acid from yolk platelets of *Xenopus laevis* oocytes using an ultracentrifugation density gradient. Jin et al. (2004) have assessed bovine X- and Y-bearing spermatozoa in fractions with a discontinuous Percoll gradient.

Utilizing a sucrose step gradient starting with a high sucrose concentration and ending with a low sucrose concentration, we found that intracellular lipid content of M-II oocytes can be determined and used to compare differences between species. Porcine oocytes compared with murine and bovine oocytes had a lower settling sucrose buoyant density concentration. These results indicate that porcine oocytes settling at a lower sucrose concentration suggesting higher lipid content. These findings support previous studies that reported a higher concentration of lipids in porcine oocytes when compared with murine and bovine oocytes when evaluated with gas chromatography (McEvoy et al., 2000). Sturmey et al. (2003) stated that porcine oocytes were darker in appearance compared with bovine and murine oocytes, and that endogenous lipids were abundant in the porcine oocyte. Murine oocytes could not be distinguished when they were dispensed into the sucrose wash. This may be associated to their relatively low lipid content and light appearance of the oocyte for visualization (Loewenstein et al., 1964).

Various stains have been used to evaluate intracellular lipid content in oocytes and embryos. Crosier et al. (2001 and 2000) used Sudan Black to evaluate the ultrastructural of bovine zygotes and blastocysts. Recently, Nile Red has been used to specifically evaluate

lipid droplets and lipid content in murine, porcine and bovine oocytes and embryos (Leroy et al., 2005).

Leroy et al. (2005) reported that the fluorescence intensity was not significantly different among bovine oocytes stained with Nile Red at different time intervals. The fluorescence of oocytes stained at 2 hours had the same fluorescence intensity found in oocytes stained at 24 hours. Techniques based on those of Genicot et al. (2005) were used to validate the Nile Red stain protocol. We found that the optimal incubation time with Nile Red is 24 hours. The 24-hour staining period resulted in the highest amount of fluorescence emitted (154.50 ± 1.59 NRU) in M-II bovine oocytes compared with 2, 4, 8, 12 and 20 hours. These results were not in agreement with the bovine oocytes reported by Leory et al. (2005).

Nile Red stain has been used to specifically stain for lipid content in oocytes. Different oocytes obtained from mice, pigs and cattle have been evaluated with Nile Red to determine the difference in intracellular lipids (Genicot et al., 2005). Eggs from worms (*Caenorhabditis elegans*) have also been stained with Nile Red to evaluate fat storage droplets (Fei et al., 2004).

Staining oocytes of mice, pigs and cattle with Nile Red for 24 hours can be used to determine the neutral lipid content in M-II oocytes (Leory et al., 2005). Porcine oocytes emitted a higher fluorescence than murine and bovine oocytes after staining with Nile Red. These results support data reported by Genicot et al. (2005). Genicot et al. (2005) reported a significant difference in fluorescence emitted in porcine oocytes when compared with murine and bovine oocytes. In the experiment, it was concluded that porcine oocytes have a higher content of intracellular lipids compared with murine and bovine oocytes based on mean fluorescence values of Nile Red.

CHAPTER IV

COMPARISON OF LIPID CONTENT IN MATURE OOCYTES FROM BRAHMAN AND ENGLISH BREEDS USING SUCROSE BUOYANT DENSITY GRADIENT AND NILE RED STAINING

INTRODUCTION

Recently, BSE was discovered in the United States, which has subsequently banned the export of live cattle to other countries. The ban has had an economical impact on US cattle producers. Cattle breeders from Mexico and South America are increasing the genetic potential of their cattle for quality of meat and heat tolerance thus, are major buyers of Brahman and Brahman-influenced cattle from the United States. The ban on live animal export has resulted in an increased demand for cryopreserved Brahman embryos from the United States.

Today, most embryo recovery and transfer procedures are nonsurgical, and more than 50% of the collected cattle embryos are cryopreserved (Thibier, 2000). Pregnancy rates often range from 50 to 60% from embryos frozen with the conventional slow-rate freezing protocols (Niemann, 1991). However, pregnancy rates from fresh transfers are generally higher when compared with frozen-thawed embryo transfers (Leibo et al., 1993; Massip, 2001). Studies have suggested that the decline in pregnancy rates may be due, at least partially, to the presence of intracellular lipids in the embryo (Hasler et al., 2001).

Bovine oocytes and embryos are difficult to cryopreserve due to chilling injury, which may be associated with intracellular lipids (Seidel, 2006). The main site of direct chilling injury during cryopreservation is within cytoplasmic membranes (Horvath et al., 2006). Intracellular lipids in the membrane go through a transition from a liquid crystalline phase to a gel phase as temperatures decrease. These phase changes result in decreased membrane integrity and even cell death (Seidel, 2006).

Nagashima et al. (1994) reported that the difficulty in cryopreserving porcine embryos is associated with intracellular lipids. As noted in porcine embryos, the low percentage of viable bovine embryos after cryopreservation may also be associated with high level of intracellular lipids. Razek et al. (1998) and McEvoy et al. (2000) reported that triglycerides are the most abundant form of fatty acids present in porcine and bovine embryos. Fatty acid levels in porcine embryos are ~2.5-fold greater than the amount of cellular lipids in bovine and ovine oocytes ($63 \pm 6 \mu\text{g}$ and $89 \pm 7 \mu\text{g}$, respectively) (McEvoy et al., 2000).

Studies on embryo lipids have demonstrated that bovine embryos with a dark ooplasm have a poor cryotolerance and result in low pregnancy rates (Hill et al., 1998). Pregnancy rates from cryopreserved bovine embryos are reported to be variable among breeds, and it is hypothesized that the lower pregnancy rates in some breeds are correlated with the high cytoplasmic lipid content of their embryos (Visintin et al., 2002, Steel et al., 2004; Looney, unpublished observations).

In general, embryos with a larger amount of lipid droplets respond poorly to cryopreservation (Seidel, 2006). The transfer of frozen *Bos indicus* and *Bos taurus* embryos produced different pregnancy rates (Visintin et al., 2002; Steel et al., 2004). Visintin et al. (2002) concluded that bovine embryo freezability is closely linked to the quality of the embryo and this differs between breeds, based on pregnancy rates comparisons made with frozen-thawed *Bos indicus* and *Bos taurus* embryos. It has been proposed that *Bos indicus* embryos have higher lipid content when compared with *Bos taurus* embryos (Looney, unpublished observations). Although the effect of breed on frozen-thawed embryo transfer pregnancy rates has been reported, limited studies have been conducted to establish if such variability is due to differences in embryo intracellular lipid content.

The main objective of this study was to determine if mature metaphase-II (M-II)

Brahman oocytes have a higher intracellular lipid content than English breeds, using a sucrose buoyant density gradient and Nile Red staining. To our knowledge, this is the first study comparing intracellular lipid content of bovine oocytes from *Bos taurus* and *Bos indicus* breed types.

MATERIALS AND METHODS

Experimental Animals

Oocyte donors were provided from local registered cattle breeders in the College Station, TX (Central Texas) area to be used in the experiment. The nonlactating cows were transported to Ovagenix (Navasota, TX) and were maintained on common bermudagrass pasture and fed 2% of total body weight 2 times daily with a 12% protein and 4% fat feed ration (Producers CO-OP, College Station, TX) (Appendix J) in addition to free choice grass hay, trace mineral mix and fresh water. Donors in each replication of this study were started on feed 25 days prior to experiment and continuing through the oocyte collection interval. This study was executed in 2 replicates (Replicate I and II) starting in the month of August.

Experimental Design

Lipid content of M-II oocytes from Brahman and English breed (Replicate I) and Brahman and English breed donors (Replicate II) were evaluated for lipid content using a sucrose buoyant density gradient and Nile Red staining. In Replicate I, the Brahman donor females had lower body condition scores, while English breed donor females had higher body condition scores. In Replicate II, Brahman donors had higher body condition scores and the English breed donor females had lower body condition scores.

Breed parameters including age, weight, body condition score (BCS) and temperament were recorded. In addition, blood samples were collected to evaluate

total circulating cholesterol and triglyceride levels in all donors twice prior to oocyte collections. Transvaginal ultrasound-guided oocyte aspiration (TUGA) was performed either 2 or 3 times from each donor in Replicates I and II, respectively.

Experimental Procedures

The age of Brahman (n=4) and Hereford (n=3) donors ranged from 4 to 6 years (Replicate I) and for the Brahman (n=7) cows and Angus (n=10) cows 5 to 10 years (Replicate II). The body weights recorded for the Brahman and Hereford donors in Replicate I ranged from 411 to 618 kg. The Brahman and Angus donors in Replicate II ranged in body weight from 361 to 750 kg. BCS for the Brahman and Hereford donors in Replicate I ranged from 4.5 to 7 and 4 to 7.5 for the Brahman and Angus donors in Replicate II. The temperament score for Brahman and Hereford donors in Replicate I ranged from 1 to 3 and the Brahman and Angus donors in Replicate II ranged from 1 to 2 in temperament score.

When all Brahman donors from Replicate I and II were combined into one group (Brahman breed group, Group 1), their ages ranged from 4 to 9 years of age. When the Hereford and Angus donor females were combined from Replicate I and II into one group (English breed group, Group 2) their ages ranged from 5 to 10 years of age. Body weights for the Brahman group ranged from 411 to 743 kg and 361 to 618 kg in the English breed group. The body condition scores (BCS) for the Brahman group ranged from 4 to 7.5 and 4 to 7 for the English breed group. In the Brahman group, temperament ranged from 1 to 3 and a temperament score of 1 was consistent within the English breed group.

Oocytes were collected twice with a 13-day interval in Replicate I and oocytes were collected 3 times at 30-day intervals in Replicate II. Cows in Replicate I were synchronized and superstimulated starting in the month of August followed by the superstimulation and aspirations in Replicate II donors. Superstimulated donors were aspirated on the morning of

day 8 of treatment. The oocytes were harvested from the Brahman and English breed donors by transvaginal ultrasound-guided oocyte aspirations (TUGA). M-II oocytes were then evaluated for lipid content with a sucrose buoyant density gradient. Nile Red staining was subsequently performed on the harvested oocytes after using buoyant density at the Embryo Biotechnology Laboratory (EBL) (Louisiana State University Agriculture Center, Baton Rouge, LA).

The open donor cows were synchronized by placing an Eazi-Breed CIDR[™] (Controlled Intravaginal Releasing Device; Pharmacia & Upjohn Company, Kalamazoo, MI), each containing 1.38 g of progesterone, into the vagina and administering an intramuscular (IM) injection of 2 ml of progesterone with estradiol (Combo, 25 mg/ml of P₄ + 1.25 mg/ml E₂; Ovagenix, Bryan, TX) (Figure 4.1). The first injection of FSH was administered on day 4 of treatment (P₄/E₂ + CIDR[™] = day 0) 2 times a day for 3 consecutive days in decreasing doses (Figure 4.1) (Appendices G and H). In Replicate I, Brahman and Hereford donors received a total of 132 and 102 mg of FSH, respectively, over the 3-day interval. Brahman and Angus donors in Replicate II received a total of 232 and 280 mg of FSH, respectively, over the 3-day interval.

Prior to the treatment of CIDR insertion and oocyte aspiration procedures, 8 to 10 ml of blood was collected via coccygeal to be analyzed for total triglycerides and cholesterol. Serum analyses were performed by the Texas Veterinary Medical Diagnostic Laboratory System (TVDMML, College Station, TX).

Oocyte Donor Collections

TUGA was performed in each donor by an experienced technician. Individual donors were restrained with a Pearson[™] working chute (Pearson Livestock Equipment, Thedford, NE). Then 6 ml (20 mg/ml) of lidocaine (2% lidocaine hydrochloride; Pets, Inc., Canton, TX)

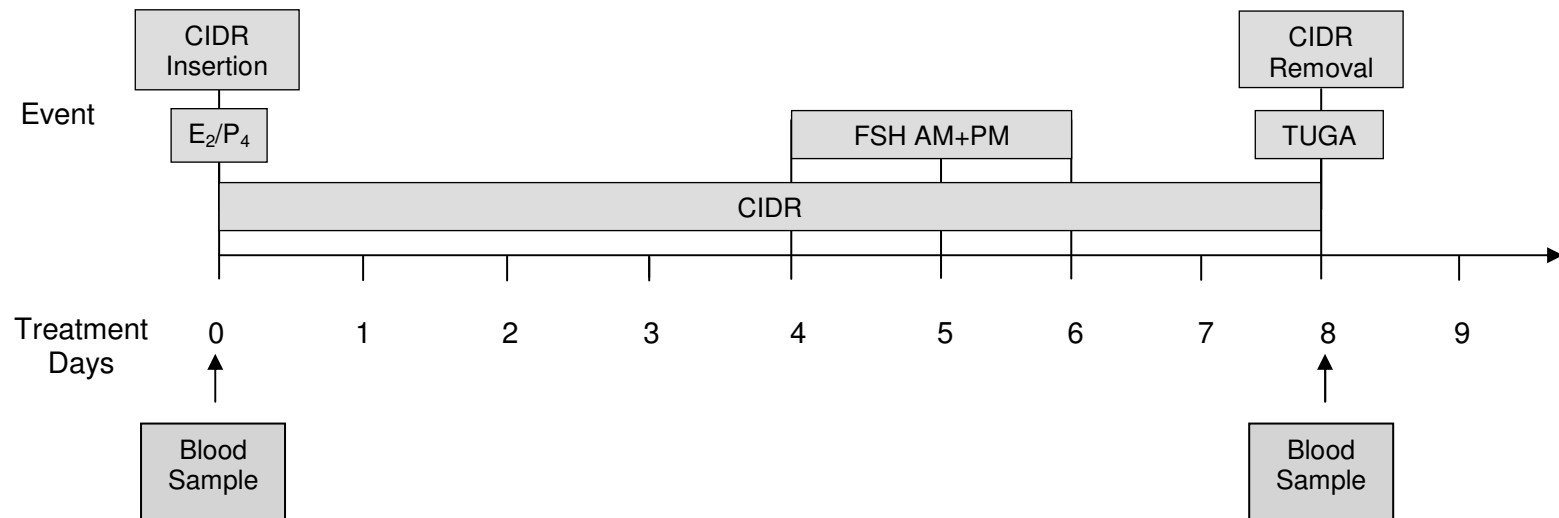


Figure 4.1. Timeline of superstimulation protocol for transvaginal ultrasound-guided aspiration (TUGA) of donor oocytes (day 8). Blood samples were collected on day 0 of treatment (CIDR insertion) and day 8 of treatment (CIDR removal and oocyte collection).

was administered into the sacral vertebrate using a 18-gauge 3.81 cm needle attached to a Monoject syringe (Pets, Inc., Canton, TX). The tail of the donor was secured by wrapping the tail switch with latex rubber tubing (VWR International, West Chester, PA) attached to the left side of the working chute. Once the tail was secured, the perineal region was rinsed free of any debris.

Ovaries of donors were stabilized rectally with the left hand, while the right hand was used to insert a 5 MHz transducer attached to a 500-mm extended handle equipped with a needle guide used for collecting oocytes. The probe and transducer were covered with a sterile latex cover prior to the beginning of each aspiration procedure. The transducer was connected to an Aloka SSD-500V portable convex sector-linear scanner (Aloka Company, Ltd., Wallingford, CT) for visualization of the ovaries and follicles for aspiration (Appendix N).

The ovaries, stabilized by the hand, were drawn to a position just lateral to the external os of the cervix. The transducer was positioned ipsilateral to the grasped ovary adjacent to the cervical os (Appendix O). From this position, the follicles (≤ 20 mm in diameter) on the ovary were viewed via the monitor of the Aloka SSD-500V (Appendix P). Prior to each aspiration, follicles on each ovary were counted and classified as small (2 to 3 mm), medium (4 to 8 mm) or large (9 to 19 mm). Follicles >20 mm in diameter were classified as follicular cysts and were aspirated after the conclusion of the initial oocyte collection procedure.

A 17-gauge, 60 cm single lumen needle (Ram IVF, Madison, WI) was rinsed with 70% isopropyl alcohol followed by Tyrodes Lactate-HEPES medium (TL HEPES; Cambrex Bio Science Walkersville, Inc., Walkersville, MD) by injecting the solution through the needle (20-gauge, 3.81 cm needle) attached to a 20 ml Norm-Ject syringe (Pets, Inc., Caton, TX). The needle, attached to 6.3 French Teflon (FTE) nonradiopaque tubing (Cook Veterinary

Products, Bloomington, IN) was rinsed by applying negative pressure with a K-Mar 5,000 regulated vacuum pump (Cook Australia, Eightmile Plains, Queensland, Australia).

Before each aspiration was performed, the vacuum pump was adjusted to assure uniform pressure throughout the procedure (78 psi with a flow rate of 21 ml/minute). The tubing was attached to an Em-Con filter (Immuno Systems, Spring Valley, WI), which was attached to the K-Mar 5,000 vacuum pump. Identification (ID) numbers representing each donor was recorded on the filter. The Em-Con filter was partially filled with Tryrodes Lactate-HEPES medium supplemented with 10% BSA. Sodium heparin (10 IU/ml) was added to prevent the clotting of the follicular fluid retrieved at the time of oocyte collection. TL HEPES supplemented with 10% FBS and 10 IU per ml of sodium heparin was aliquoted into 50 ml plastic conical tubes and placed in digital dry block heaters at 38°C (VWR International, West Chester, PA) next to the animal working chute.

Once the initial set-up was completed, the needle punctured the ovary and was passed through as much ovarian tissue as possible before puncturing the follicles. Puncturing follicles directly on the surface of the ovary would cause loss of the follicular fluid and thus, loss of the oocyte. Passing the needle through the ovarian tissue prior to puncturing the follicle greatly reduced the incidence of leakage and loss of oocytes.

While holding the ovary with the follicle centered on the puncture line (indicated by an arrow on the monitor) the needle was pushed into the follicle. The needle required only moderate forward pressure to be pushed into the follicle. Once the follicle was punctured, the needle was quickly placed into the follicle, and twisted 360° forward and backward while holding the transducer (Appendix Q). This prevented pockets of follicular fluid from remaining as the follicle was aspirated. The pockets of follicular fluid tend to be difficult to aspirate after the initial collapse of the follicle, and because they often harbor oocytes, it was

important that the entire fluid be aspirated during the first follicle puncture. To prevent blockage of the tubing and loss of oocytes in blood clots, the needle was either withdrawn back into the needle guide and allowed to aspirate air or totally removed from the needle guide to rinse with TL HEPES buffer. The needle was then reinserted into the ovary at a different location and additional follicles were aspirated. The oocyte retrieval session started in the morning at 8:00 a.m., with the first donor female was usually completed within 20 to 30 minutes from the time the female was restrained.

After all follicles ranging in size from 2 to 20 mm were aspirated, the needle was removed from the needle guide, and the tip was placed in the remaining TL HEPES buffer solution. Vacuum was applied until all the remaining follicular fluid in the needle or tubing was aspirated into the filter. The tubing was disconnected from the filter and brought inside the laboratory to be rinsed. The tail was released and the donor exited the animal working chute.

The filter containing the aspirated oocytes was taken into the laboratory and rinsed with TL HEPES medium previously placed in a heated water bath (VWR International, West Chester, PA) at 38 °C. The small amount of aspirate remaining in the EM-Con filter was poured into a 100 x 15 mm disposable, sterile, intergrid-square petri dish (VWR International, West Chester, PA). A 50 ml Norm-Ject syringe was filled with TL HEPES solution and a 20-gauge, 3.81 cm needle was attached. Moderate pressure was applied to the syringe plunger to expel the solution with a gentle jet stream to facilitate separation of the collection fluid from the cumulus oocyte complexes (COCs). The sides and mesh of the filter were thoroughly rinsed with this stream. While rinsing, the filter was held as to allow the solution to drain into the search dish. Each individual donor's ID number was recorded on the dish. The dish was set aside to allow the contents to settle and foam to dissipate before searching for the

oocytes. COCs in the dish were identified and evaluated using an inverted Zeiss stereoscope (Carl Zeiss, Inc., Thuringia, Germany) at 10X magnification.

In Vitro Maturation

COCs were removed from the search dishes and placed in a 35 x 10 mm disposable, sterile petri dish (VWR International, West Chester, PA) of Tissue Culture Medium-199 with Earles salts (TCM-199; Gibco BRL, Grand Island, NY). All oocytes from individual donors were located and graded from a scale of A to E with respect to their cumulus cell status as follows: A = compact cumulus (>5 layers) and normal cytoplasmic appearance, B = 3 to 4 cumulus layers with a compact cumulus complex, C = 1 to 2 cumulus layers with any slight cytoplasmic abnormalities, D = denuded and E = expanded cumulus with gross cytoplasmic abnormalities (Looney et al., 1994).

After 4 rinses in TCM-199, the oocytes were matured in TCM-199 supplemented with 10% heat-inactivated characterized fetal bovine serum (Hyclone Laboratories, Logan, UT), 0.01 units per ml of both bFSH and bLH (Sioux Biochemical, Sioux City, IA) (Appendix I). Oocytes were subsequently grouped by donor and matured for 20 hours in 35 x 10 mm disposable, sterile petri dishes (Falcon[®], Becton Dickinson and Company, Franklin Lakes, NJ) in a humidified auto flow water-jacketed incubator (Nuair 4950 US, Biomedical Solutions, Stafford, TX) at 38.5°C with 5% CO₂ in air. After maturation, cumulus cells were removed by vortexing at high speeds for 3 minutes with 1 ml of Tyrode HEPES in a 15 ml plastic conical tube.

Sucrose Buoyant Density Measurement

Only oocytes with a distinct polar body (M-II stage oocytes) were used in this experiment. Visual color scores of 1, 2 and 3 (light, medium and dark) were assigned to these M-II oocytes prior to measurement in the sucrose buoyant density gradient. Sucrose

buoyant density gradient measurements were performed and recorded on individual donor oocytes. Briefly, fresh sucrose medium was prepared in sterile 50 ml centrifuge tubes. First, 0.5 ml of sucrose medium was added per level in a 5 ml culture test tube (VWR International, West Chester, PA). Gradient steps ranged from 23 to 35% sucrose (oocytes settling in 23% sucrose have higher lipid content than oocytes settling at 35%) (Appendix D). Each level was carefully dispensed in descending order, so that each level would not be disrupted.

Mature oocytes used for sucrose buoyant density measurements were washed 2 times in Dulbecco's PBS and then 2 times in Dulbecco's PBS with 23% sucrose (top sucrose level). Washing oocytes in sucrose prior to taking measurements allowed them to settle through the gradient. The oocytes were removed from the sucrose wash using a glass pipette. Oocytes were then gently dispensed on to the top layer of the gradient. Usually 10 oocytes were measured per gradient. Oocytes were viewed as they settled through the gradient using a Zeiss stereoscope (Carl Zeiss, Inc., Thuringia, Germany) placed horizontally (Figure 3.4). The oocytes were allowed to settle for 1 hour and were individually recovered from their respective level using a glass pipette. Oocytes were grouped from the layer at which they settled and washed 2 times in Dulbecco's PBS.

Nile Red Staining

Oocytes recovered from the sucrose buoyant density gradient were incubated in 0.25 ml plastic, sterile straws (Agtech, Inc., Manhattan, KS) (1 to 10 oocytes per straw) containing a Glutaraldehyde/Formaldehyde fixative medium (Appendix E). Fixed oocytes were held at 4 °C for 24 hours and were transported in an insulated container on ice to Louisiana State University Embryo Biotechnology Laboratory (EBL) (Louisiana State University Agriculture Center, Baton Rouge, LA).

Immediately upon arrival to the EBL, oocytes were recovered from the 0.25 ml plastic

straws and placed into a 35 x 10 mm disposable sterile, petri dish (Falcon[®], Becton Dickinson and Company, Franklin Lakes, NJ). Oocytes were evaluated using a Nikon SMZ-2B stereoscope (Nikon, Inc., Melville, NY) and recovered via a glass pipette. The M-II stage oocytes were placed into a sterile 4-well Nunc dish (Nunc, Intermed, Roskilde, Denmark) and washed 4 times with Dulbecco's PBS. Donor oocytes were kept separate based on sucrose settling level during the wash. Matured oocytes were incubated in droplets of Nile Red solution (Appendix F) in a 35 x 10 mm petri dish in total darkness at room temperature (24 °C) for 24 hours inside a sealed container.

Oocytes were recovered and washed 2 times in Dulbecco's PBS in a sterile 4-well Nunc dish after incubation with the Nile Red. Oocytes were suspended in 10 µl of Invitrogen ProLong[®] Gold antifade reagent (Molecular Probes, Inc., Eugene, OR) on a 25 x 55 mm glass slide. Then 3 to 4 oocytes were placed in each droplet. A cover slip was placed over the ProLong[®] Gold droplet and sealed with nail polish.

Evaluation of oocytes stained with the Nile Red (550 to 800 fluorescence emission) was performed using a Nikon Optiphot microscope (Nikon, Inc., Melville, NY) equipped with epifluorescent illumination and an FITC dichroic filter cube. Images of each oocyte were captured with phase and fluorescence microscopy, using a Hitachi D20b camera attached to the microscope and stored on a PC computer using Epix software. After images were captured, Scion Image software (Scion Corporation, Frederick, MD) was used to evaluate the mean fluorescence intensity of the Nile Red staining in the oocytes. Higher Nile Red Units (NRU) indicates higher lipid content and lower NRU indicates lower lipid content present in the M-II stage oocyte. Scion Image uses a 8 bit grey scale to evaluate fluorescence intensity.

Cholesterol and Triglyceride Concentrations

Blood samples were collected on the day of CIDR insertion (treatment day 0) and on the day of CIDR removal for each oocyte collection per donor (oocyte aspiration on day 8). Restraint was applied with the animal working chute to donors to ensure quick, easy and safe collection of the sample causing minimal stress. Collection of blood was performed by puncturing the tail vein (coccygeal) with a 20-gauge x 1A Monoject blood collection needle attached to a vacutainer hub. A sterile sodium heparin tube (Becton Dickinson, Franklin Lakes, NJ) was used for collecting the samples. For each donor, 8 to 10 ml of blood was collected and stored at 8°C for 24 hours.

The blood samples were centrifuged at 2,400 rpm for 15 minutes with an ALC Model OK120 (Nantes, Western France) to separate serum from the cells. After centrifugation was completed, serum was aspirated and placed in polyethylene scintillation vials. Vials containing the serum were stored at -20°C until samples for all groups were collected. The serum was then transported to the Texas Veterinary Medical Diagnostic Laboratory System (TVDMML, College Station, TX) to be analyzed for total circulating cholesterol and triglyceride concentrations.

Statistical Analysis

Data for this study were processed using SAS software Version 9.1.3 (SAS Institute Inc., Cary, NC) and analysis was performed using LS-Means. Differences between treatments were considered significant at the 0.05 significance level. Variables evaluated include: mean oocyte color score, mean oocyte sucrose settling concentrations and mean fluorescence of oocytes from different breed types, high and low body condition score, mean circulating cholesterol and mean circulating triglyceride concentration.

RESULTS

Replicate I: Brahman vs. Hereford

The mean number of follicles aspirated/donor and oocytes recovered/donor in Replicate I were 20.0 and 11.2 oocytes for the Brahman donor females and 10.0 follicles aspirated/donor and 7.0 oocytes/donor for the Hereford donor group.

The overall mean sucrose buoyant density value (percent) when all aspirations were combined for M-II stage Brahman oocytes was $31.3\% \pm 0.19$ compared with Hereford M-II stage oocytes at $31.4\% \pm 0.37$. Sucrose settling point of M-II stage oocytes was not different between breed types when all oocytes were combined for oocyte aspirations ($n=2$) in Replicate I (Figure 4.2). However, there were significant differences ($P < 0.05$) between breed type for oocytes for aspiration 1 and aspiration 2. In addition, no significant differences were detected between Brahman and Hereford donors in aspiration 1 and aspiration 2 (31.1 ± 0.12 to 30.6 ± 0.19 and 31.6 ± 0.46 to $32.1\% \pm 0.69$) (Figure 4.3).

The mean fluorescence intensity in the Brahman and Hereford M-II stage oocytes (aspiration 1 and 2 combined) after Nile Red staining was 115 ± 7.4 and 114 ± 15.1 Nile Red Units (NRU), respectively. No significant difference ($P < 0.05$) was detected when the aspirations were combined (aspiration 1 and 2) within both breed types (Figure 4.4). The mean fluorescence for Brahman and Hereford M-II oocytes from aspiration 1 was not significantly different ($P < 0.05$) (106 ± 9.8 and 87 ± 17.5 NRU). Furthermore, in aspiration 2, no significant difference in M-II stage oocytes stained with Nile Red was detected between the 2 different breed types (124 ± 11.3 and 151 ± 16.4 NRU, respectively). However, significant differences ($P < 0.05$) were detected in fluorescence intensity of M-II stage oocytes from the Brahman and Hereford donors when aspiration 1 was compared with aspiration 2 (107 ± 9.8 to 151 ± 16.4 and 87 ± 17.5 to 124 ± 11.3 , respectively) (Figure 4.5).

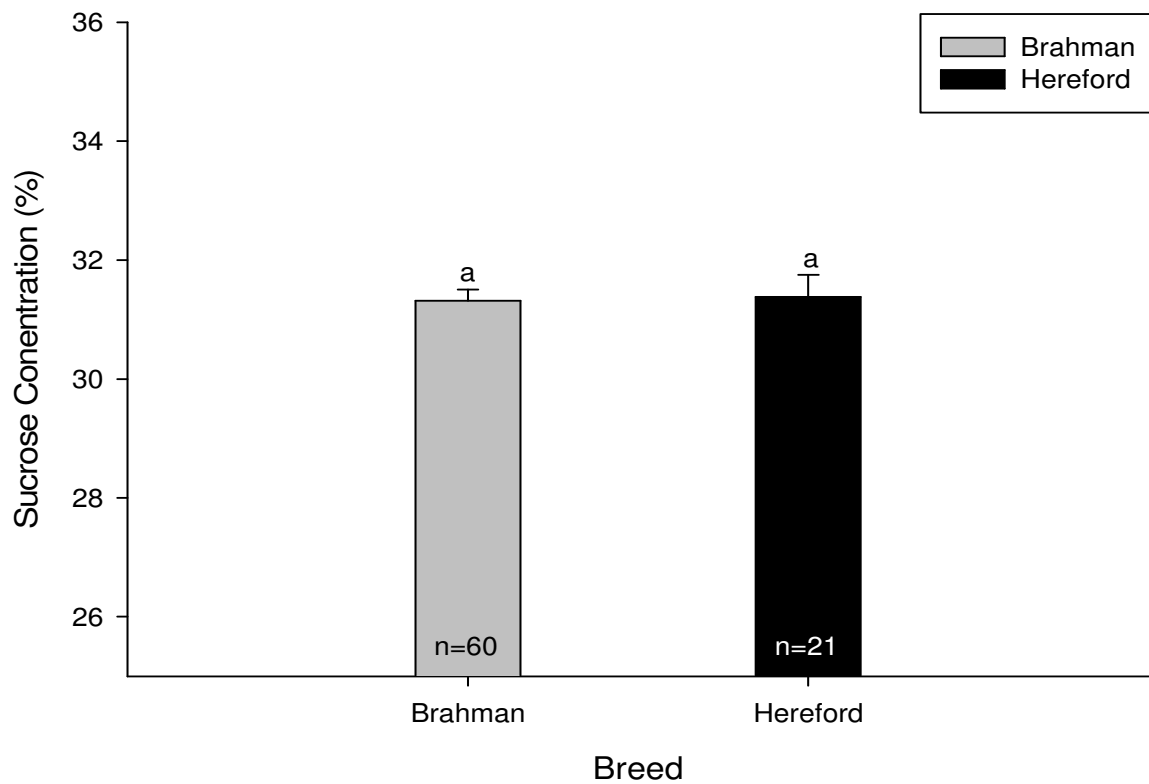


Figure 4.2. Mean (\pm SEM) sucrose buoyant density values of Brahman and Hereford M-II stage oocytes in various concentrations of sucrose. ^{a,b}Means with different letters between breeds are significantly different ($P < 0.05$).

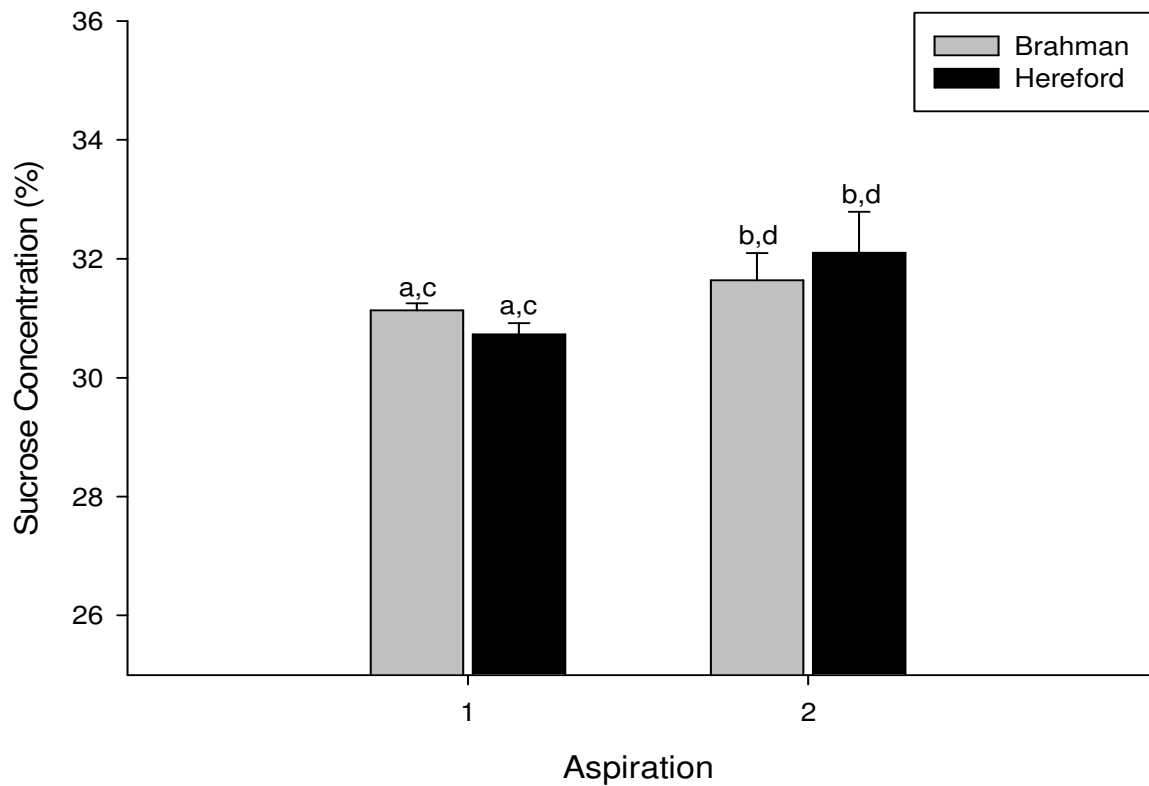


Figure 4.3. Sucrose buoyant density (mean \pm SEM) values of Brahman and Hereford M-II stage oocytes grouped by aspirations. ^{a,b}Means with different letters within aspirations are significantly different ($P<0.05$). ^{c,d}Means with different letters between aspirations within breeds are significantly different ($P<0.05$).

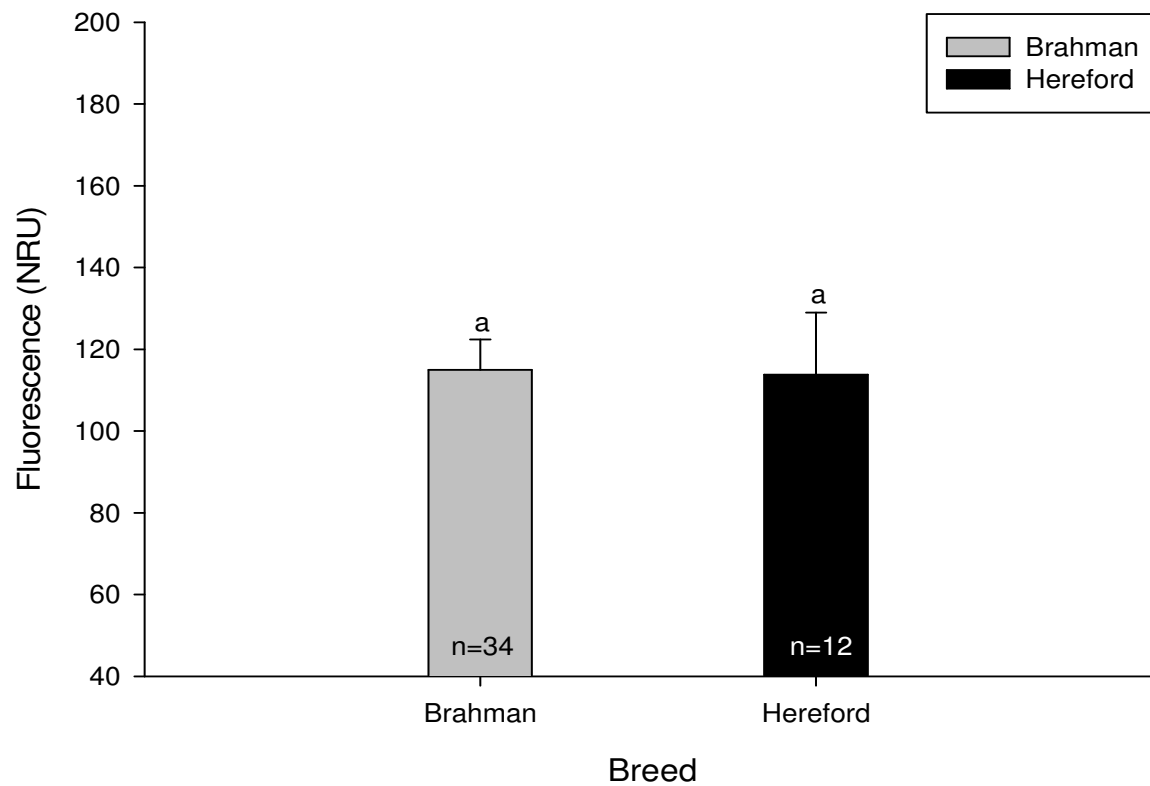


Figure 4.4. Mean (\pm SEM) fluorescence intensity values of Brahman and Hereford M-II stage oocytes after sucrose buoyant density measurements. ^{a,b}Means with different letters between breeds are significantly different ($P < 0.05$).

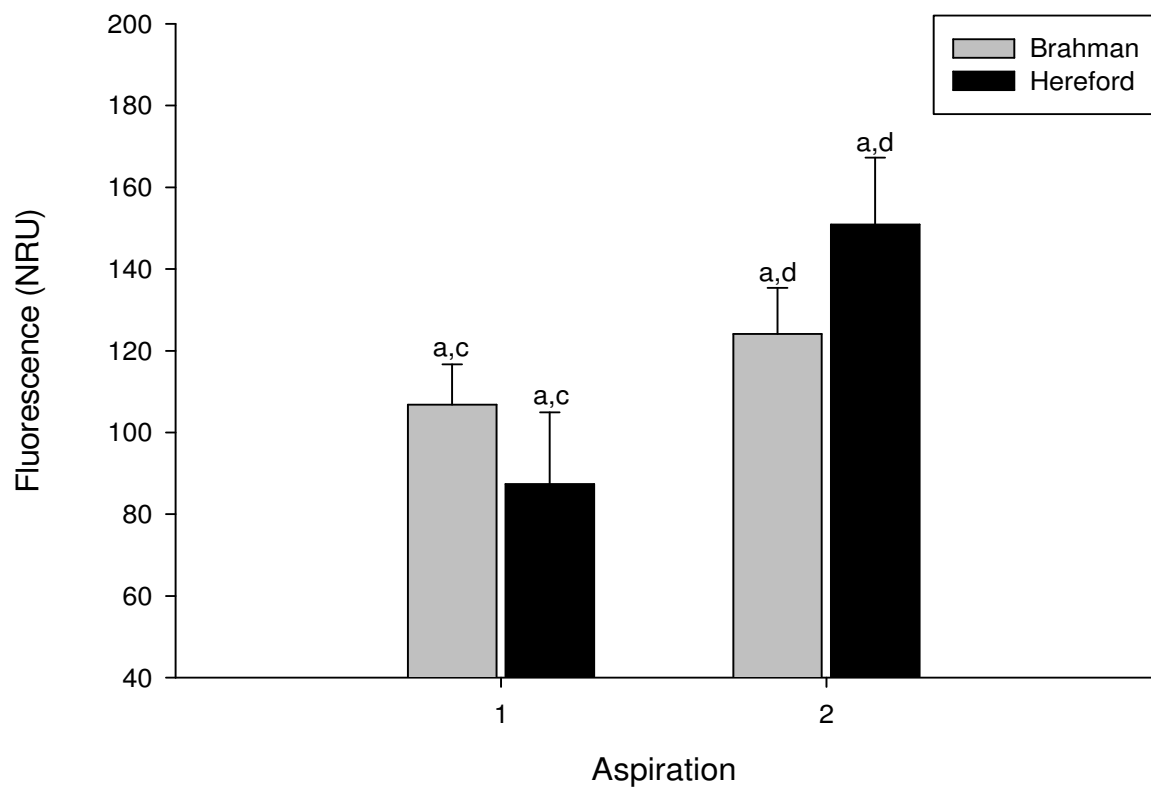


Figure 4.5. Mean fluorescence intensity (mean \pm SEM) values of Brahman and Hereford M-II stage oocytes after sucrose buoyant density measurements grouped by aspirations. ^{a,b}Means with different letters within aspirations are significantly different ($P<0.05$). ^{c,d}Means with different letters between aspirations within breeds are significantly different ($P<0.05$).

Circulating cholesterol and triglyceride concentrations were measured in Brahman and Hereford donors prior to CIDR insertion and CIDR removal. The cholesterol concentration at the time of CIDR insertion and CIDR removal was 156.5 ± 20.73 and 147.5 ± 18.64 mg/dl, respectively, for Brahmans at aspiration 1. Mean circulating cholesterol concentration of Brahmans at aspiration 2 was 157 ± 17.40 and 129.25 ± 18.56 mg/dl for CIDR insertion and CIDR removal. There were no significant differences ($P < 0.05$) between aspiration 1 and aspiration 2 when comparing the concentration of cholesterol at CIDR insertion to CIDR removal in the Brahman donor females (Figure 4.6).

Hereford donors mean circulating cholesterol concentrations at CIDR insertion compared with CIDR removal at aspiration 1 was 109.3 ± 13.17 to 13.7 ± 10.40 mg/dl and 119.2 ± 12.22 to 94 ± 20.79 mg/dl at aspiration 2. No significant differences ($P < 0.05$) were detected for circulating cholesterol concentration between CIDR insertion and CIDR removal at both aspiration 1 and aspiration 2 for the Hereford donor females (Figure 4.7). When comparing the overall mean circulating cholesterol concentrations at CIDR insertion and CIDR removal grouped by aspiration, the Brahman donors females had a higher cholesterol concentration than Hereford donor females in aspiration 1. However, no significant difference was detected between the 2 breeds at aspiration 2 for circulating cholesterol concentrations combined at the time of CIDR insertion and CIDR removal (Figure 4.8).

The mean circulating cholesterol concentrations at CIDR insertion for both oocyte aspirations were 156.8 ± 12.53 in Brahman donors and 114.2 ± 8.32 mg/dl for Hereford donors. The mean cholesterol levels for Brahman and Hereford donors when the CIDR removed vales were combined for both aspirations were 138.8 ± 12.65 and 98.8 ± 10.62 mg/dl, respectively, for each group. There was significant differences ($P < 0.05$) in circulating

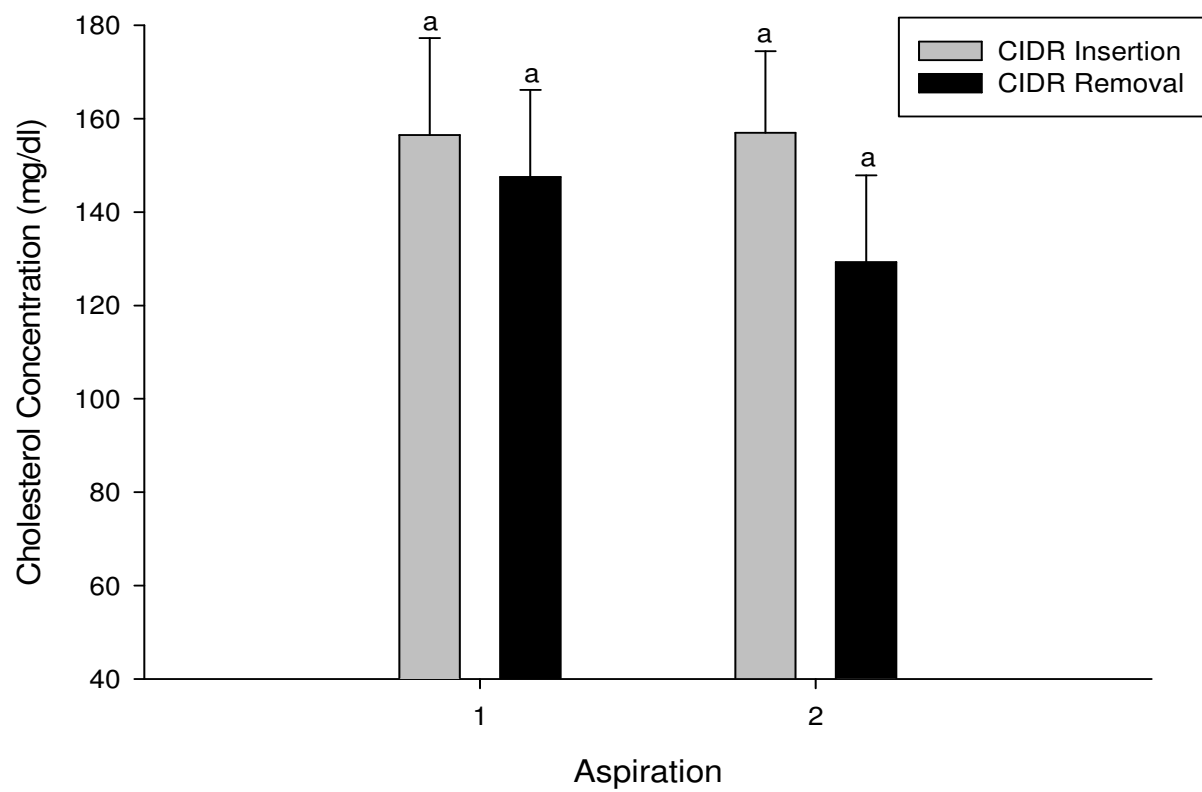


Figure 4.6. Mean cholesterol concentrations at CIDR insertion and CIDR removal (mean \pm SEM) of Brahman donors. ^{a,b}Means with different letters within and between aspirations are significantly different (P<0.05).

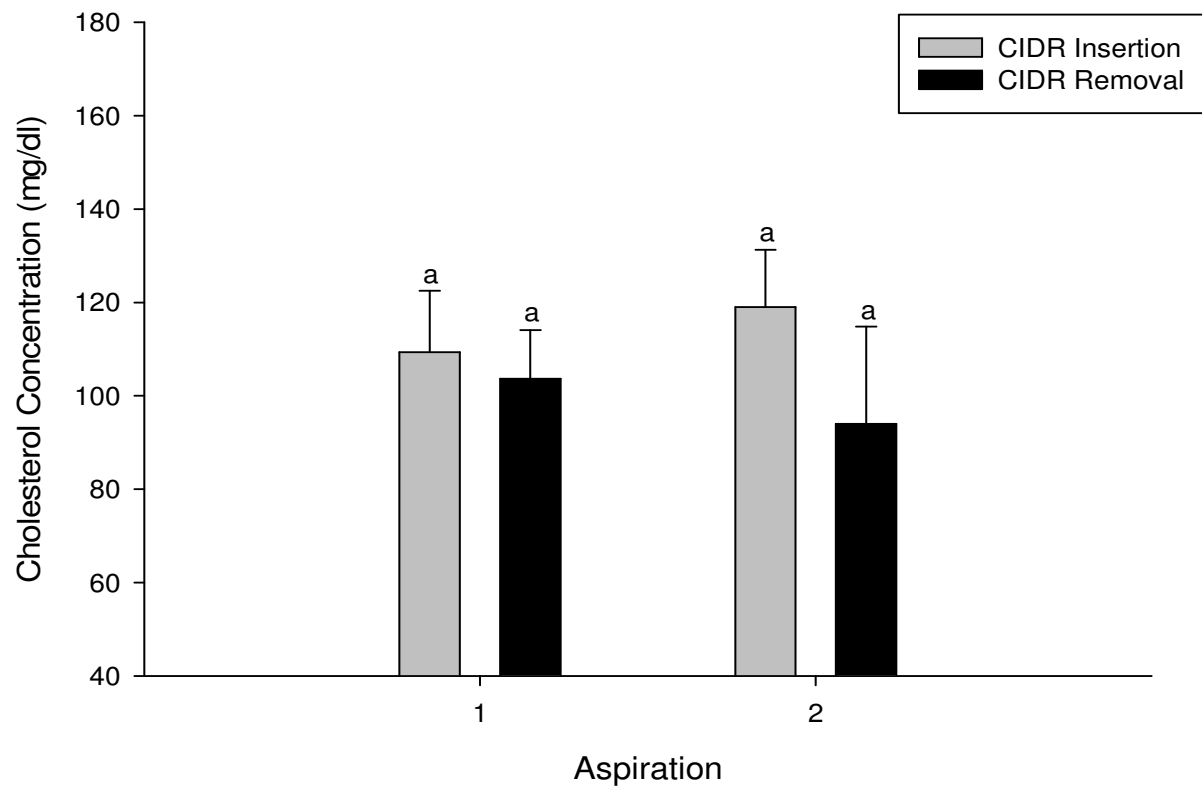


Figure 4.7. Mean cholesterol concentrations at the time of CIDR insertion and CIDR removal (mean \pm SEM) of Hereford donors. ^{a,b}Means with different letters within and between aspirations are significantly different (P<0.05).

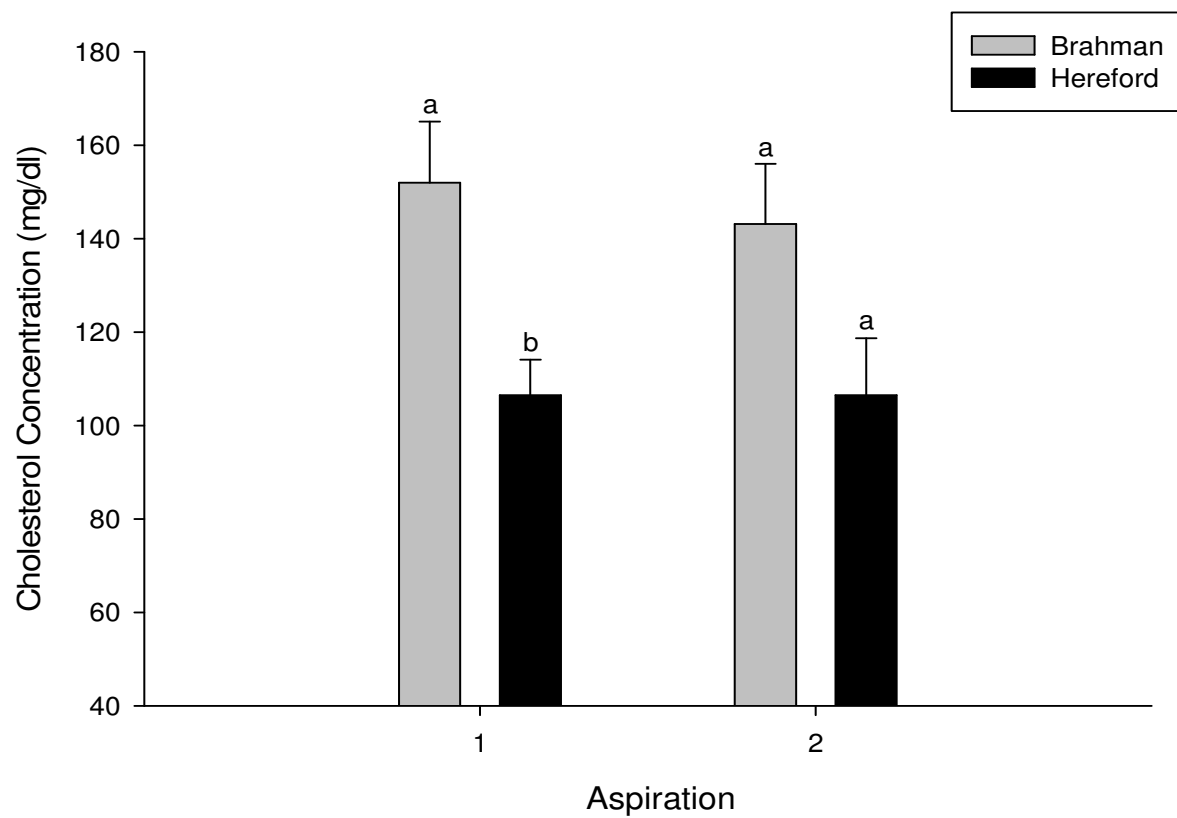


Figure 4.8. The overall mean cholesterol concentrations at CIDR insertion and CIDR removal (mean \pm SEM) of Brahman and Hereford donors grouped by aspirations. ^{a,b}Means with different letters within aspirations are significantly different ($P < 0.05$).

cholesterol for both breed types for CIIDR insertion and CIDR removal values (Figure 4.9)

Mean circulating triglyceride concentrations for the Brahman donors at the time of CIDR insertion compared with CIDR removal values for aspiration 1 (29.5 ± 4.35 and 21.8 ± 1.89 mg/dl, respectively) was not significantly different ($P > 0.05$). However, a significant difference ($P < 0.05$) was found for aspiration 2 between mean CIDR insertion values and mean CIDR removal values (39 ± 3.49 and 22 ± 3.49 mg/dl, respectively) for the Brahman donors. In addition, there was a significant difference ($P < 0.05$) between mean CIDR insertion values between aspiration 1 and aspiration 2 for Brahman donors. No significant difference was found between aspiration 1 and aspiration 2 when CIDR removal values were compared for the Brahman donor females (Figure 4.10).

The mean circulating triglyceride concentration for Hereford donors at the time of CIDR insertion and CIDR removal in aspiration 1 were 20.3 ± 7.31 and 19.4 ± 3.50 mg/dl, respectively. At aspiration 2, the mean triglyceride concentrations for the Hereford donors were 44.3 ± 12.25 and 23.3 ± 3.48 mg/dl at the time of CIDR insertion and CIDR removal, respectively. No significant differences ($P < 0.05$) were detected between mean CIDR insertion values and mean CIDR removal values for both aspirations 1 and aspiration 2 in Hereford donor females (Figure 4.11).

The overall mean circulating triglyceride concentrations in Brahman donors at CIDR insertion and at CIDR removal combined by aspirations were 25.6 ± 2.63 and 30.5 ± 2.63 mg/dl, respectively. Hereford donors had an overall mean circulating triglyceride concentrations at aspiration 1 of 19.7 ± 3.64 and 33.8 ± 7.38 mg/dl at aspiration 2. There was no significant difference between breeds when CIDR insertion values and CIDR removal values were combined for aspiration 1 and aspiration 2. In addition, no significant differences was detected when comparing mean overall circulating triglyceride

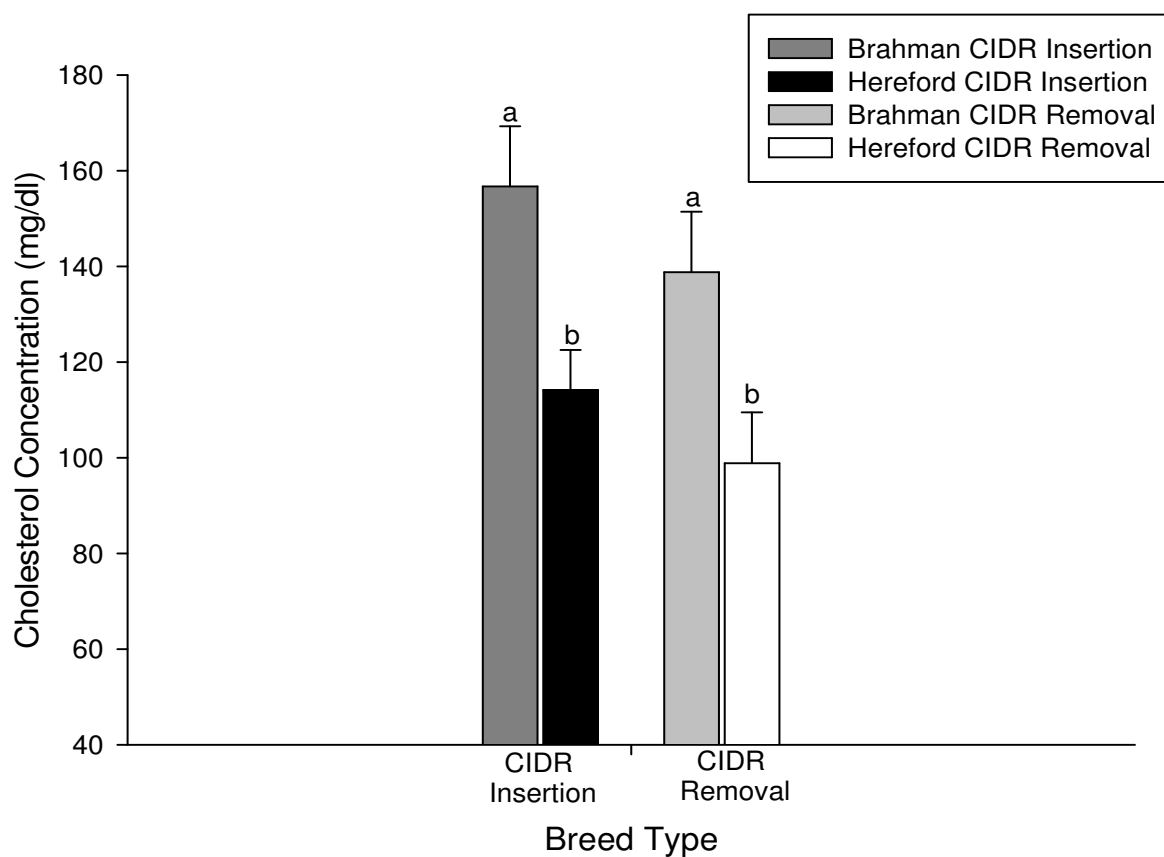


Figure 4.9. Mean (\pm SEM) cholesterol concentrations at CIDR insertion and CIDR removal in Brahman and Hereford donors. ^{a,b}Means with different letters within breeds are significantly different ($P < 0.05$).

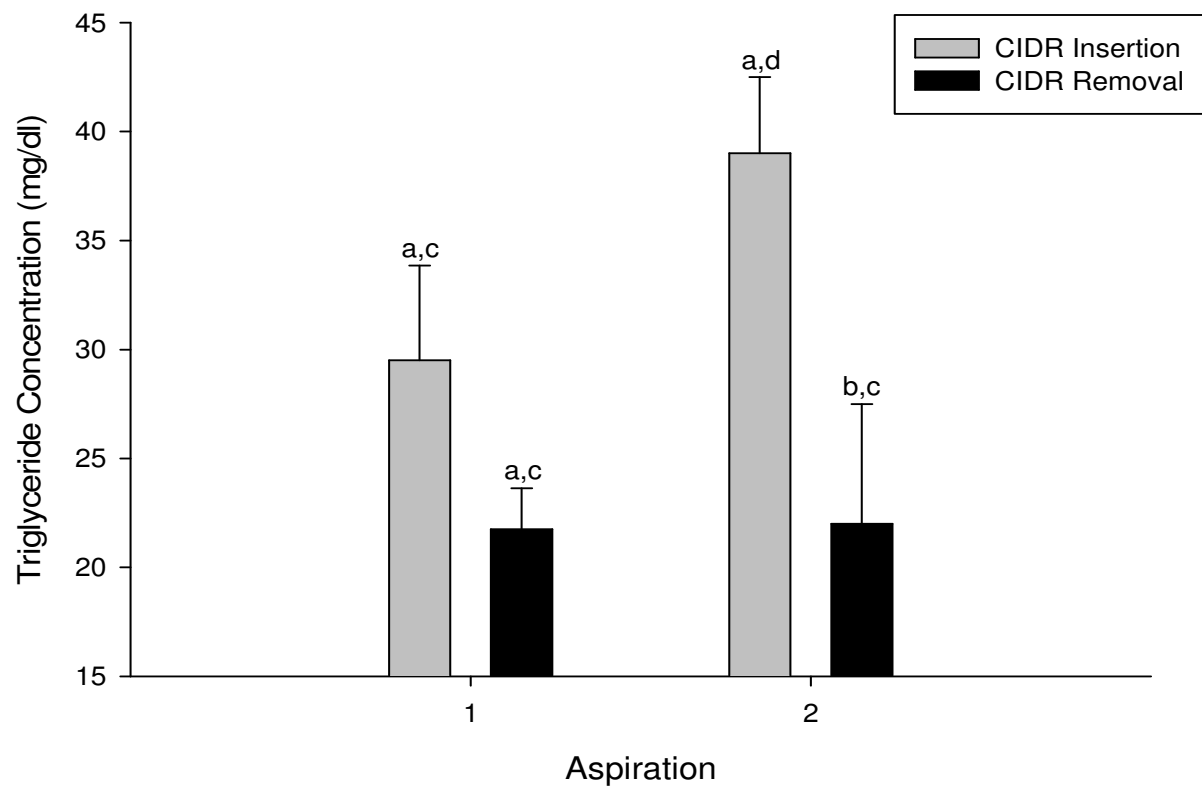


Figure 4.10. Mean triglyceride concentrations at CIDR insertion and CIDR removal (mean \pm SEM) in Brahman donors. ^{a,b}Means with different letters within aspirations are significantly different ($P<0.05$). ^{c,d}Means with different letters between aspirations within breed are significantly different ($P<0.05$).

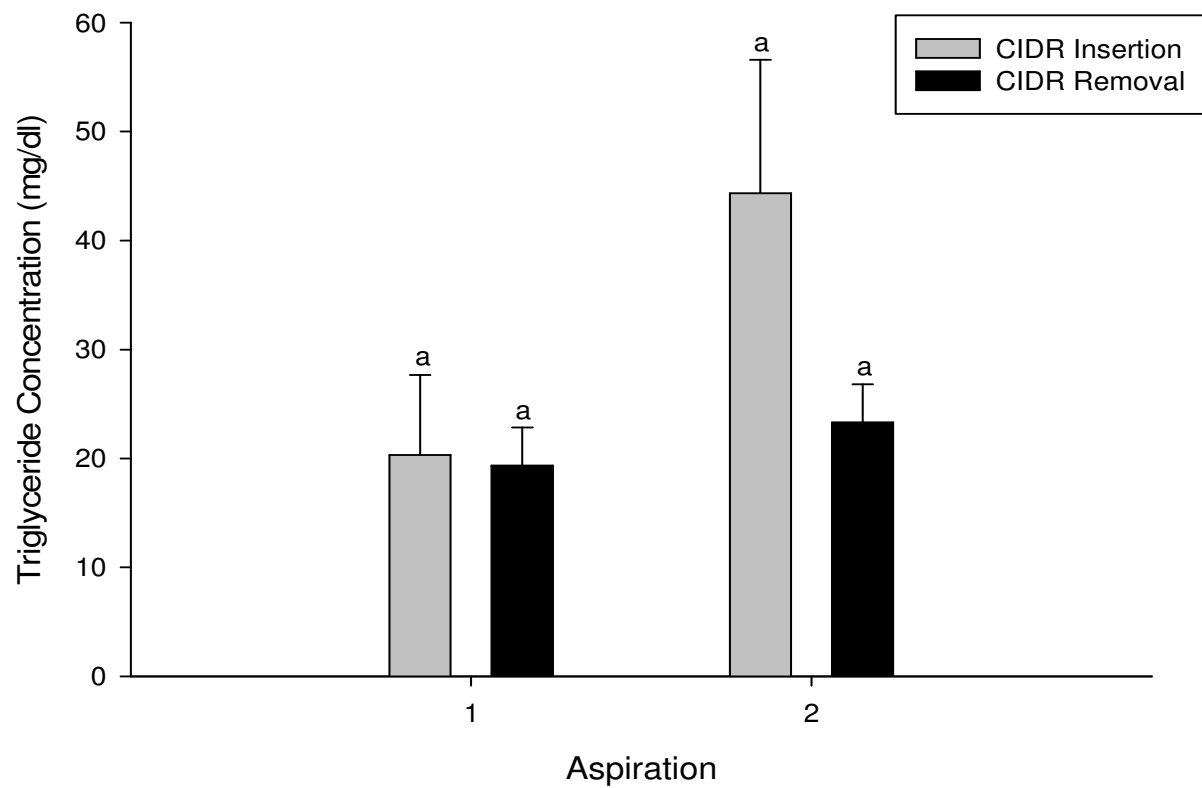


Figure 4.11. Mean triglyceride concentrations at CIDR insertion and CIDR removal (mean \pm SEM) in Hereford donors grouped by aspirations. ^{a,b}Means with different letters within and between aspirations are significantly different ($P<0.05$).

concentrations at aspiration 1 to aspiration 2 for both Brahman and Hereford donor females (Figure 4.12).

The overall mean circulating triglyceride concentrations in Brahman donors at CIDR insertion for both aspirations were 34.3 ± 3.14 and 32.3 ± 8.30 mg/dl for the Hereford donors. The overall mean triglyceride concentrations from both aspirations for Brahman and Hereford donors was 21.87 ± 2.70 and 21.2 ± 2.14 mg/dl, respectively, at the time of CIDR removal. There were no significant differences in circulating triglyceride concentrations for both breed types at the time of CIDR insertion and CIDR removal (Figure 4.13). However, the circulating triglyceride concentrations did decrease whenever the CIDRs were removed in both breed types.

Replicate II: Brahman vs. Angus

The mean number of follicles aspirated/donor and oocytes recovered/donor in Replicate II were 20.0 and 16.6 oocytes for the Brahman donor females and 12.0 follicles aspirated/donor and 7.1 oocytes/donor for the Angus donor females. M-II stage Brahman and Angus oocytes settled at $32.9\% \pm 0.10$ and $33.7\% \pm 0.12$ in the sucrose buoyant density gradient. Significant differences ($P < 0.001$) were detected in mean sucrose settling level among aspirations ($n=3$) (Figure 4.14). The mean sucrose levels in Brahman oocytes at aspirations 1 and 2 (32.5 ± 0.21 and $32.5\% \pm 0.16$) were not significantly different, but the mean sucrose level in aspiration 3 ($32.4\% \pm 0.41$) was significantly different ($P < 0.05$) when compared with aspirations 1 and 2.

The mean sucrose level in Angus M-II stage oocytes in aspiration 1 ($33.1\% \pm 0.25$) was significantly different when compared with oocytes in aspiration 2 ($34.2\% \pm 0.14$), however, the mean sucrose level of oocytes at aspiration 3 (33.6 ± 0.21) was not significantly different when compared with oocytes in aspiration 1 (Figure 4.15). M-II oocytes from

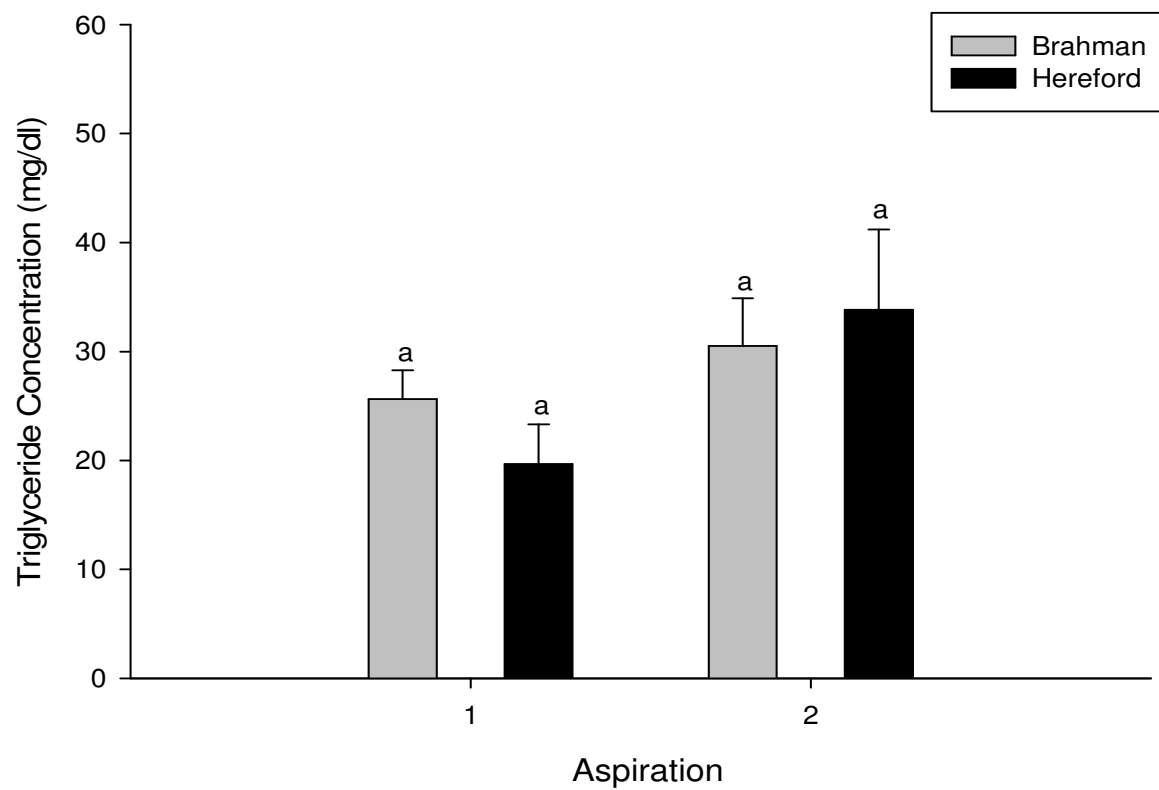


Figure 4.12. Mean triglyceride concentrations at CIDR insertion and CIDR removal (mean \pm SEM) in Brahman and Hereford donors grouped by aspirations.
^{a,b}Means with different letters within and between aspirations are significantly different ($P < 0.05$).

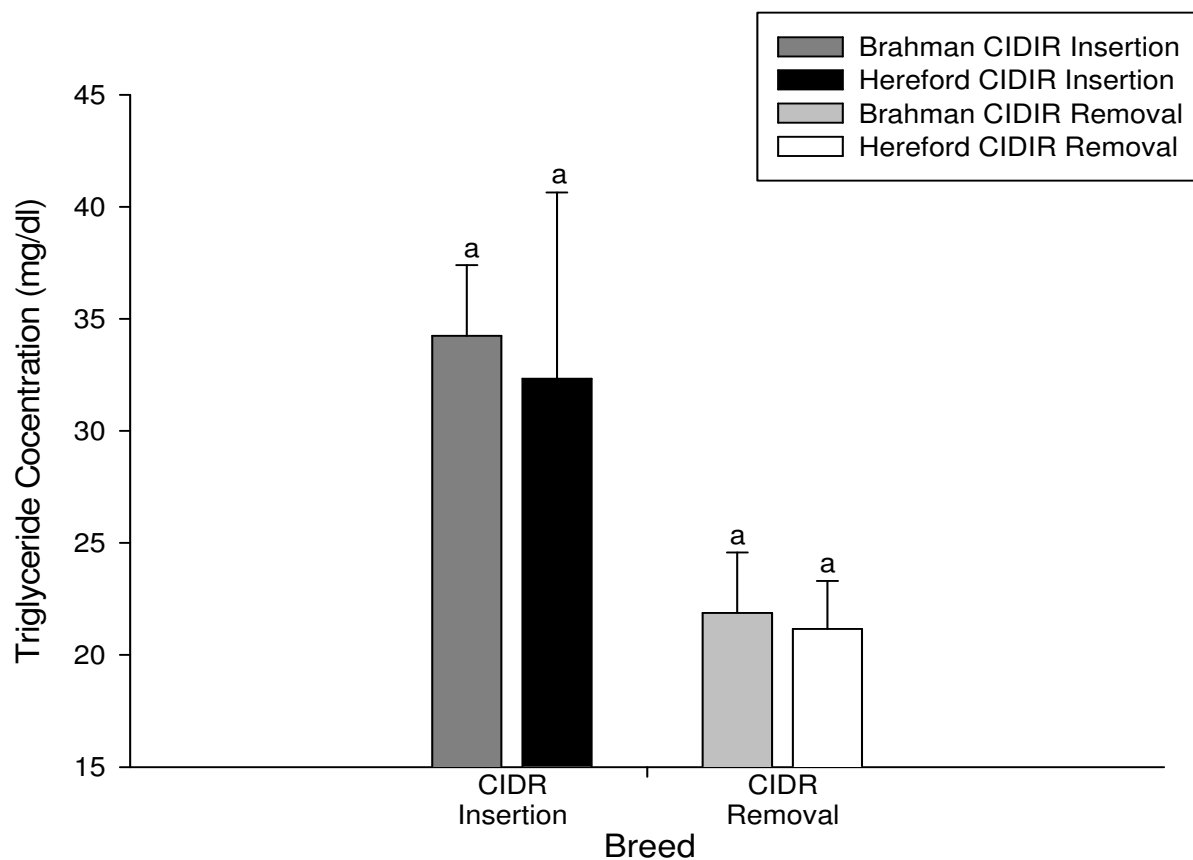


Figure 4.13. Mean (\pm SEM) triglyceride concentrations at CIDR insertion and CIDR removal in Brahman and Hereford donors. ^{a,b}Means with different letters within breeds are significantly different ($P < 0.05$).

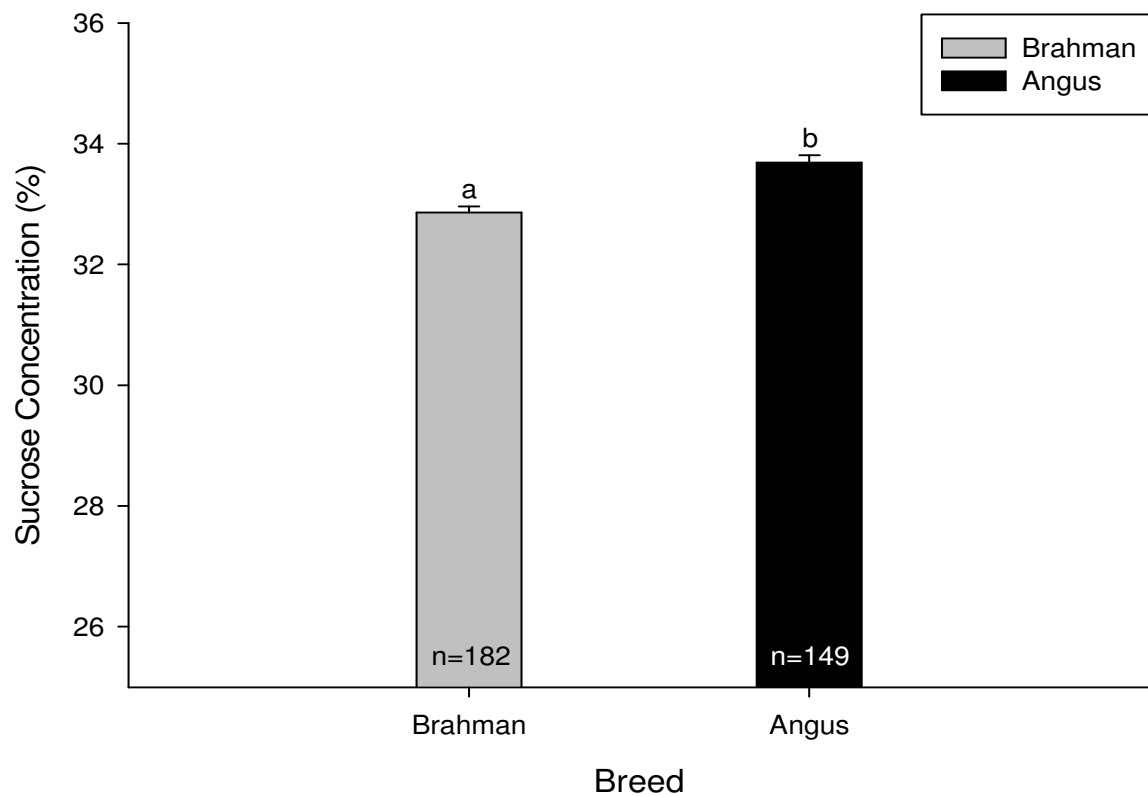


Figure 4.14. Mean (\pm SEM) sucrose buoyant density values in Brahman and Angus M-II stage oocytes. ^{a,b}Means with different letters between breed types are significantly different ($P < 0.05$).

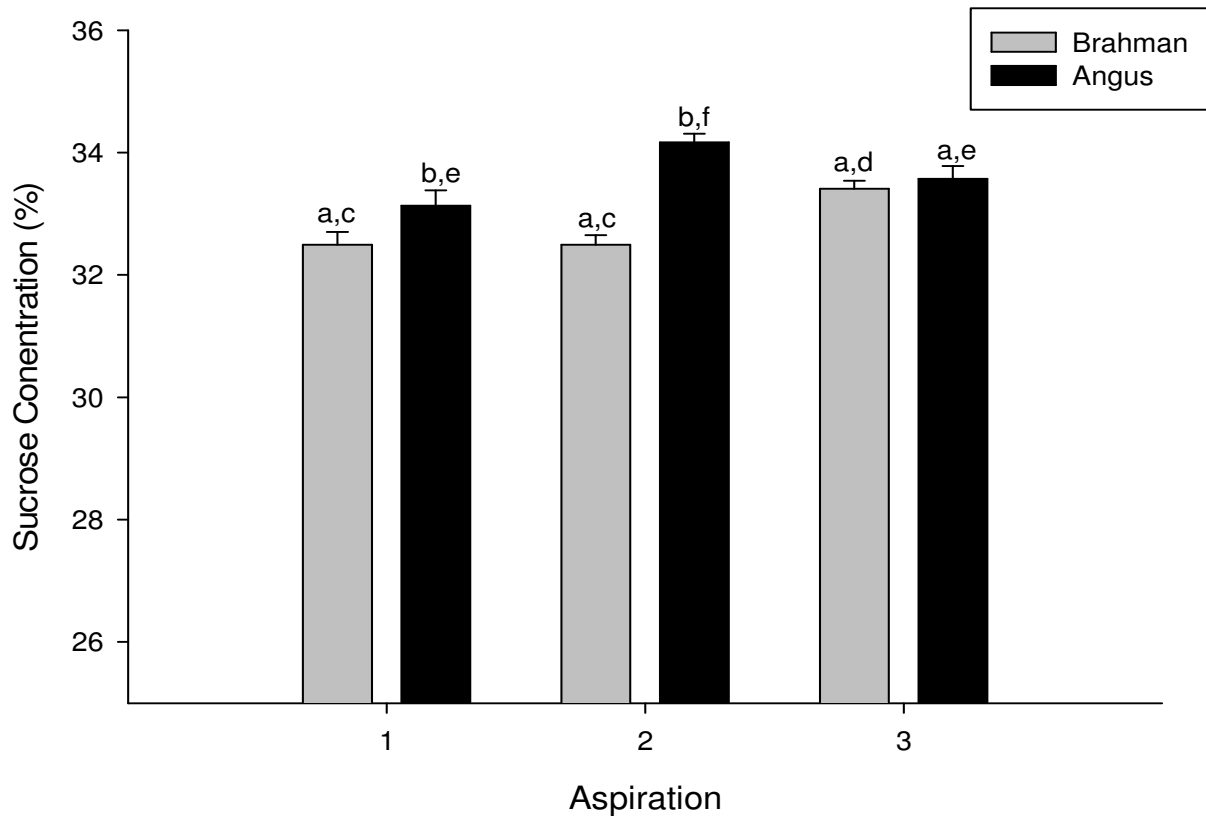


Figure 4.15. Mean (\pm SEM) sucrose buoyant density values in Brahman and Angus M-II stage oocytes grouped by aspirations. ^{a,b}Means with different letters within aspirations are significantly different ($P < 0.05$). ^{c-f}Means with different letters between aspirations within breed types are significantly different ($P < 0.05$).

Brahman donor females settled at a lower sucrose buoyant density gradient than Angus donor females oocytes in the first 2 aspirations, and these were significantly different from each other. Furthermore, there was no significant difference in aspiration 3 between Brahman and Angus M-II stage oocytes.

Overall mean Nile Red intensity values in the Brahman and Angus M-II stage oocytes were 187 ± 3.51 and 156 ± 4.41 NRU, respectively. A significant difference ($P < 0.001$) was found between breed types when the aspirations were combined (Figure 4.16). The mean fluorescence in Brahman M-II stage oocytes when compared with Angus M-II stage oocytes was significantly higher ($P < 0.01$) when aspirations ($n=3$) were combined. At aspirations 1, 2 and 3, no significant differences ($P > 0.05$) were noted in fluorescence intensity of Brahman oocytes (188 ± 8.45 , 185 ± 5.80 and 188 ± 4.40 NRU, respectively). However, Nile Red intensity in Angus oocytes at aspiration 1 (143 ± 9.44 NRU) was significantly less ($P < 0.05$) when compared with oocytes in aspiration 3 (170 ± 6.10 NRU). In addition, no significant difference was detected when Angus M-II oocytes in aspiration 1 were compared with oocytes in aspiration 2 (154 ± 6.91). Likewise, no significant difference was detected when Angus M-II stage oocytes in aspiration 2 were compared with oocytes in aspiration 3 (143 ± 9.44 and 154 ± 6.91 , respectively) (Figure 4.17).

Blood samples were collected to measure circulating cholesterol and triglyceride concentration in Brahman and Angus donor females. Overall, the mean cholesterol concentrations at the time of CIDR insertion and CIDR removal were 143.9 ± 11.7 and 141.3 ± 10.6 mg/dl, respectively, for Brahman donors at aspiration 1. Mean cholesterol concentration in Brahman donors at aspiration 2 were 162.7 ± 9.4 mg/dl at the time of CIDR removal. The mean cholesterol levels of Brahman donor females at aspiration 3 for CIDR insertion and CIDR removal was 148.7 ± 13.0 and 143.7 ± 12.1 , respectively. No significant

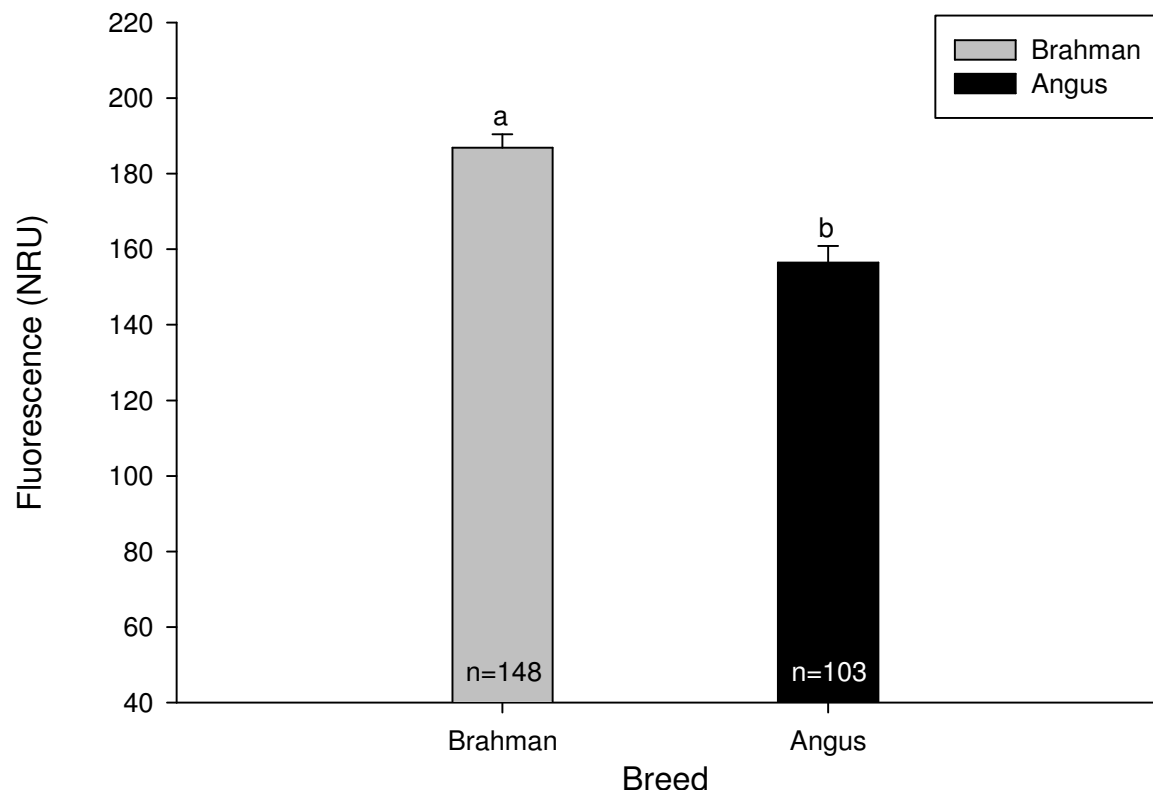


Figure 4.16. Mean (\pm SEM) fluorescence intensity values of Brahman and Angus M-II stage oocytes after sucrose buoyant density measurements. ^{a,b}Means with different letters between breed types are significantly different ($P < 0.05$).

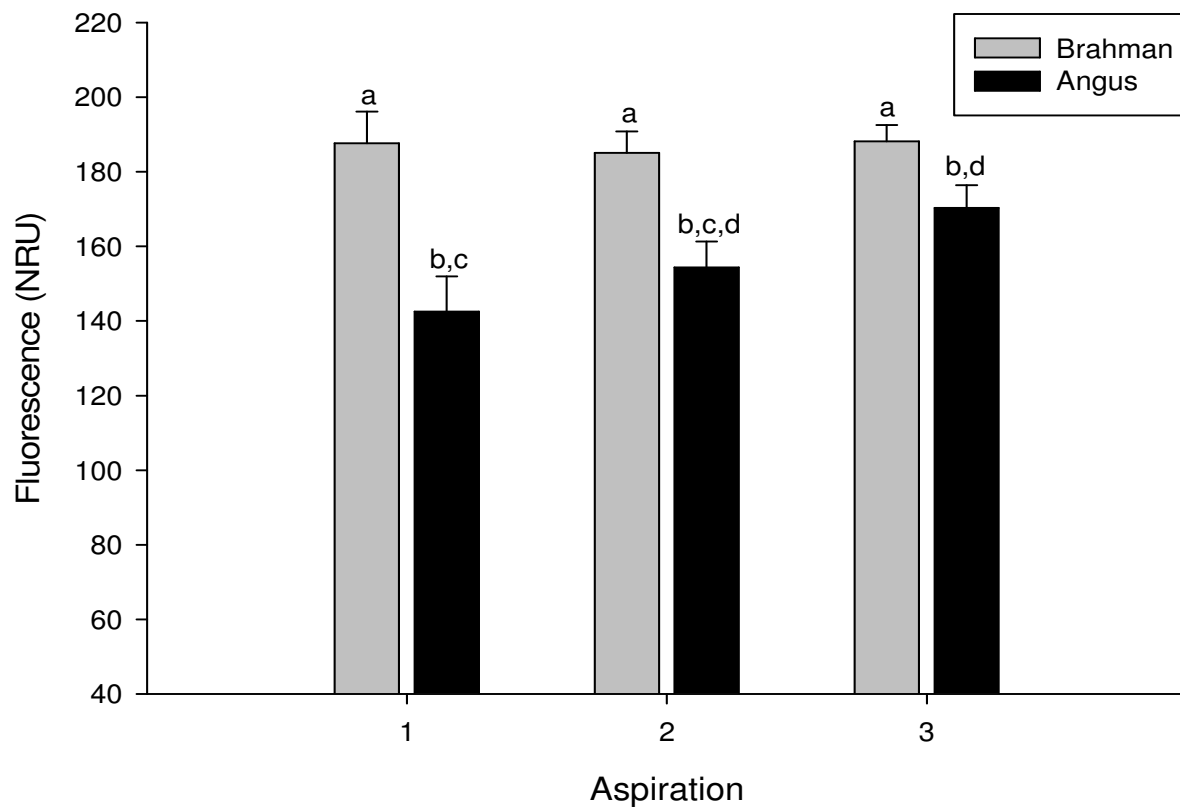


Figure 4.17. Mean (\pm SEM) fluorescence intensity values of Brahman and Angus M-II stage oocytes after sucrose buoyant density measurements grouped by aspirations. ^{a,b}Means with different letters within aspirations are significantly different ($P < 0.05$). ^{c,d}Means with different letters between aspirations within breed types are significantly different ($P < 0.05$).

differences were noted across aspirations 1, 2 and 3 when comparing cholesterol levels at CIDR insertion and CIDR removal in Brahman donor females (Figure 4.18). The serum for cholesterol evaluation at the time of CIDR insertion at aspiration 2 was mishandled and could not be analyzed for either Brahman donors or Angus donors.

Mean Angus cholesterol concentrations at CIDR insertion and at CIDR removal for aspiration 1 were 100.6 ± 7.4 and 101.3 ± 7.8 mg/dl, respectively. At aspiration 2, the mean circulating cholesterol concentration was 122 ± 5.9 mg/dl at the time of CIDR removal. The mean cholesterol concentrations in Angus donors at aspiration 3 at the time of CIDR insertion and CIDR removal were 114.1 ± 9.9 and 119 ± 10.9 mg/dl, respectively. No significant difference was detected between CIDR insertion and CIDR removal for both aspirations 1 and 3 for the Angus donor females. However, a significant increase was found when comparing the mean cholesterol levels for the Angus donor females at CIDR removal at aspirations 1 and 2 (Figure 4.19).

When comparing the overall mean circulating cholesterol concentrations at CIDR insertion and CIDR removal grouped by aspirations, a significant decrease ($P < 0.05$) was detected in aspiration 1, 2 and 3 between Brahman and Angus donor females (142.6 ± 7.6 to 101.0 ± 5.2 , 162.7 ± 9.4 to 122 ± 5.9 , and 146.2 ± 8.5 to 116.6 ± 7.1 mg/dl, respectively). However, no significant difference was detected when Brahman and Angus circulating cholesterol concentrations were compared by individual breed across aspirations 1, 2 and 3 (Figure 4.20).

The overall mean circulating cholesterol concentrations for Brahman and Angus donor females at CIDR insertion was 146 ± 8.4 and 106.2 ± 5.9 mg/dl, respectively. The mean cholesterol concentrations at CIDR removal for Brahman donor females was 149.5 ± 6.2 and 113.2 ± 4.9 mg/dl for the Angus donor females. There was a significant decrease ($P < 0.05$) in

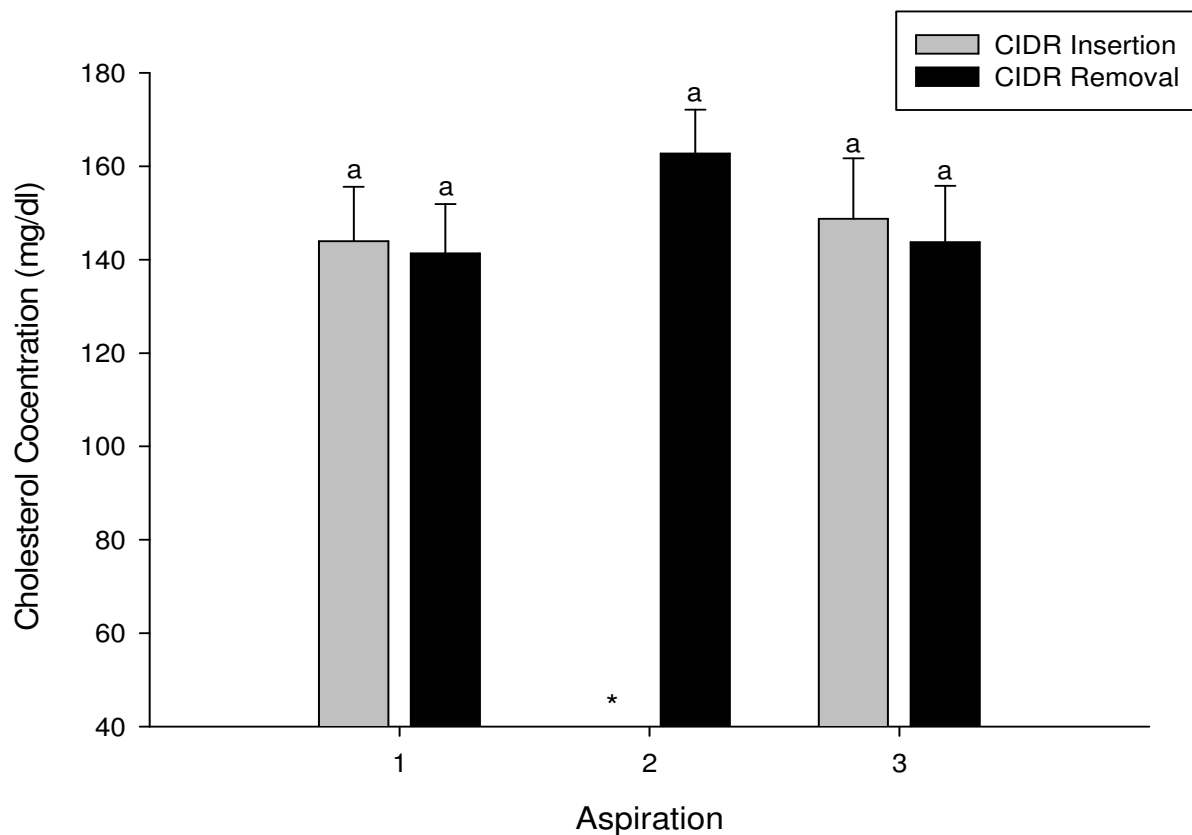


Figure 4.18. Mean cholesterol concentrations (mean \pm SEM) for Brahman donors at CIDR insertion and CIDR removal. ^{a,b}Means with different letters within and between aspirations are significantly different ($P<0.05$). *The serum at the time of CIDR insertion at aspiration 2 was mishandled and could not be analyzed for Brahman donor females.

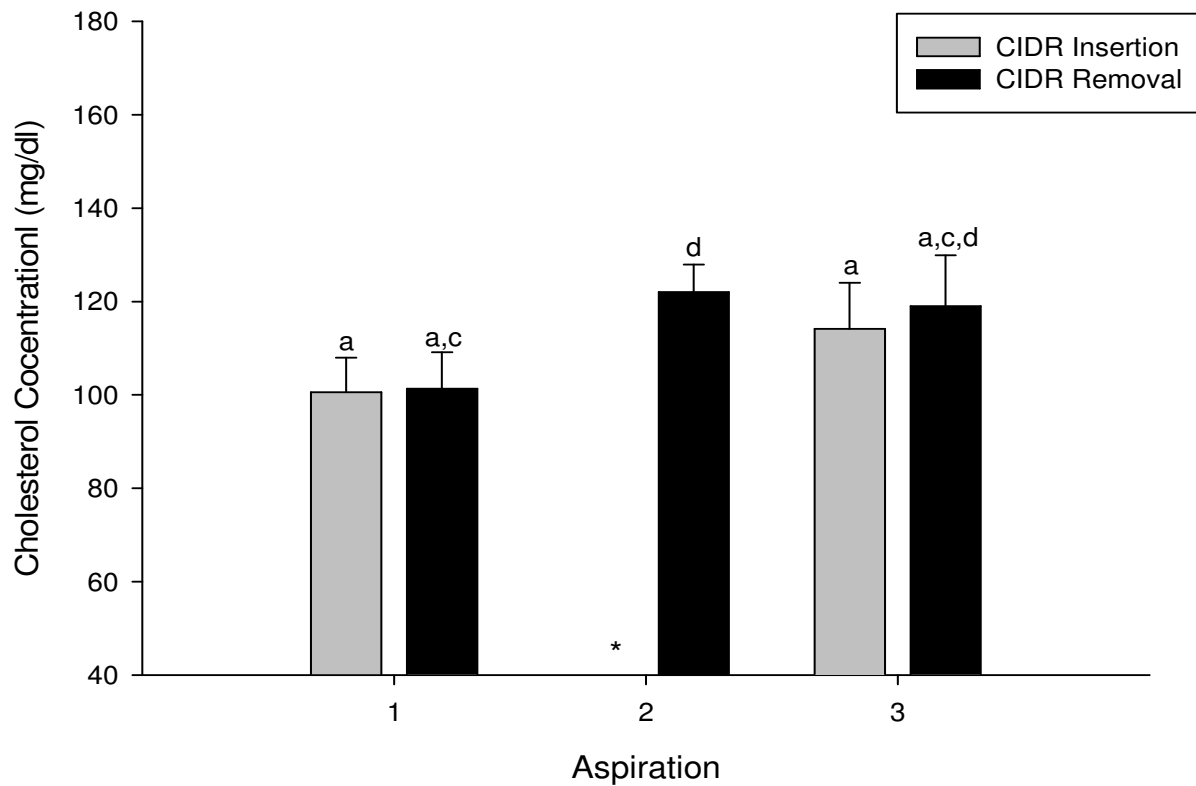


Figure 4.19. Mean (\pm SEM) cholesterol concentrations for Angus donors measured at CIDR insertion and CIDR removal. ^{a,b}Means with different letters within aspirations are significantly different ($P < 0.05$). ^{c,d}Means with different letters between aspirations are significantly different ($P < 0.05$). *The serum at the time of CIDR insertion at aspiration 2 was mishandled and could not be analyzed for Angus donor females.

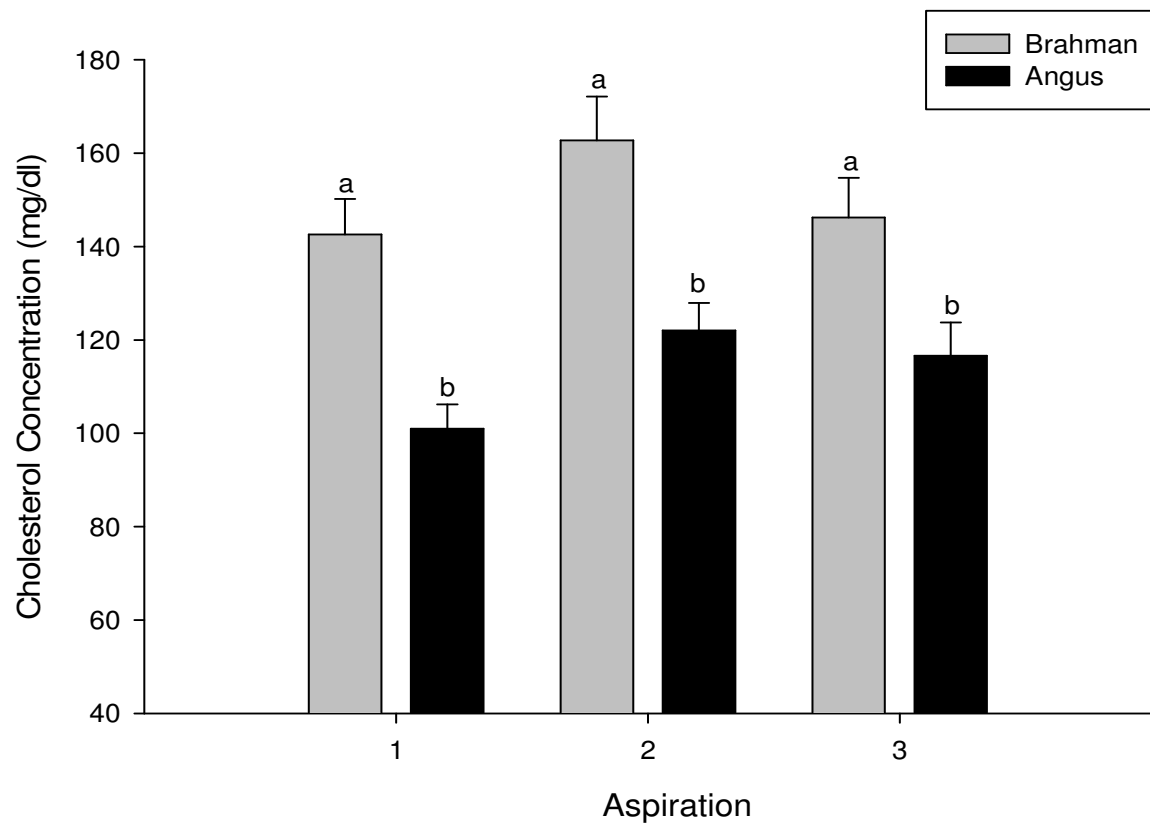


Figure 4.20. Mean (\pm SEM) cholesterol concentrations at CIDR insertion and CIDR removal in Brahman and Angus donors grouped by aspirations. ^{a,b}Means with different letters within aspirations are significantly different ($P < 0.05$).

cholesterol concentrations for both breed groups at CIDR insertion and CIDR removal (Figure 4.21).

The mean circulating triglyceride concentrations in Brahman donors at CIDR insertion compared to CIDR removal at aspiration 1 (39.9 ± 3.2 to 34.6 ± 3.8 mg/dl, respectively) were not significantly different. Significant differences could not be tested at aspiration 2 due to missing triglyceride values at CIDR insertion. The mean circulating triglyceride concentration at CIDR removal, however, was 33.9 ± 5.8 mg/dl. A significant decrease ($P < 0.05$) in triglyceride levels was detected in the Brahman donors between CIDR insertion and CIDR removal at aspiration 3 (40.5 ± 3.1 and 29.7 ± 2.6 , respectively) (Figure 4.22).

The mean triglyceride concentrations in Angus donors at aspiration 1 at the time of CIDR insertion and CIDR removal was 28.2 ± 1.3 and 23.8 ± 3.1 mg/dl, respectively. The mean triglyceride concentration at CIDR removal for Angus donors at aspiration 2 was 24.6 ± 2.8 mg/dl. The mean triglyceride concentrations at aspiration 3 at CIDR insertion and CIDR removal were 25.4 ± 1.2 and 25.4 ± 1.8 mg/dl, respectively. No significant difference was noted in triglyceride levels between CIDR insertion and CIDR removal at aspiration 1 and 3 in the Angus donor females. Furthermore, no significant difference ($P < 0.05$) was found in circulating triglyceride concentrations at CIDR insertion and CIDR removal between the aspirations (Figure 4.23).

When the overall mean circulating triglyceride concentrations in Brahman donors at CIDR insertion and CIDR removal was combined, individual serum levels were 37.2 ± 2.5 , 33.9 ± 5.8 and 35.1 ± 8.5 mg/dl, respectively. In the Angus donor females, mean overall triglyceride concentrations at aspirations 1, 2 and 3 were 26 ± 1.7 , 24.6 ± 2.8 and 24.4 ± 1.1 mg/dl, respectively. There was a significant decrease ($P < 0.05$) between the Brahman and Angus donors when CIDR insertion and CIDR removal were combined for aspirations 1 and

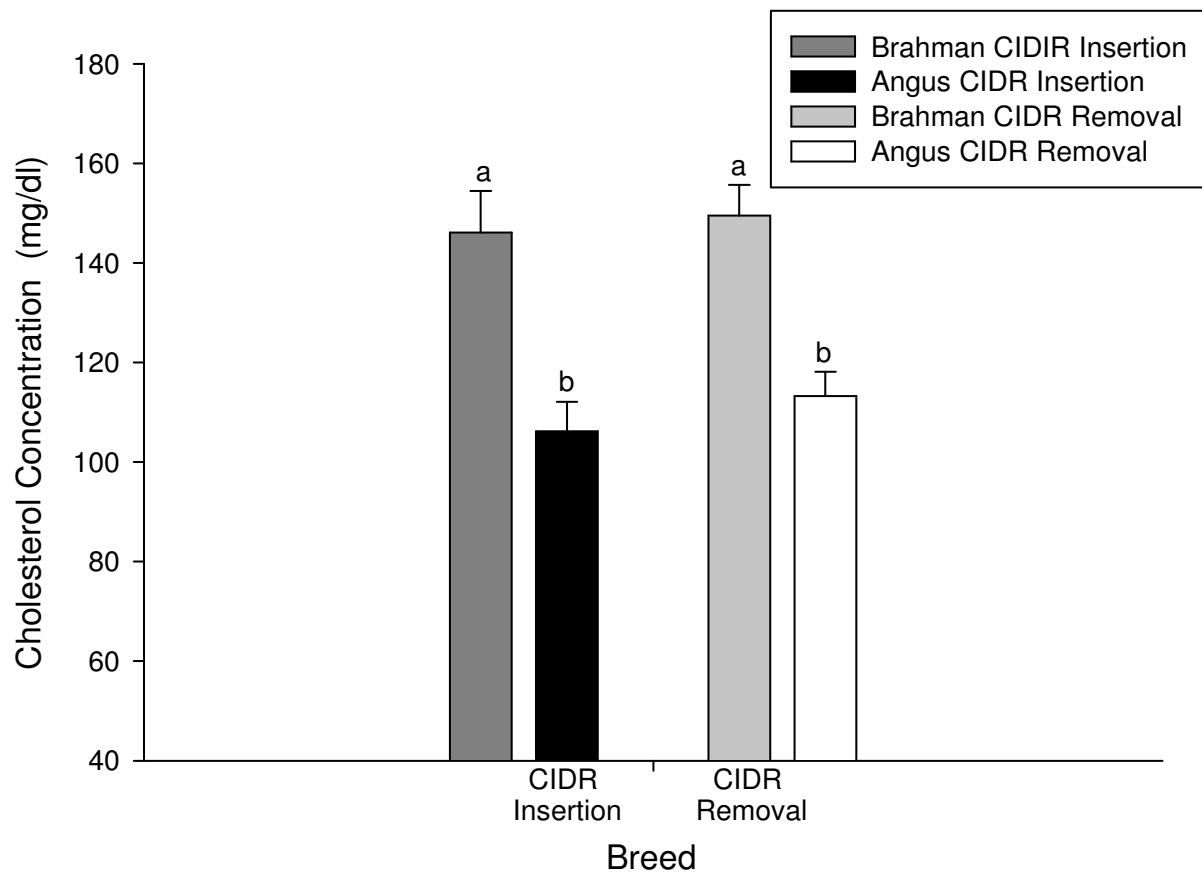


Figure 4.21. Mean (\pm SEM) cholesterol concentrations at CIDR insertion and CIDR removal in Brahman and Angus donors. ^{a,b}Means with different letters within breed types are significantly different ($P < 0.05$).

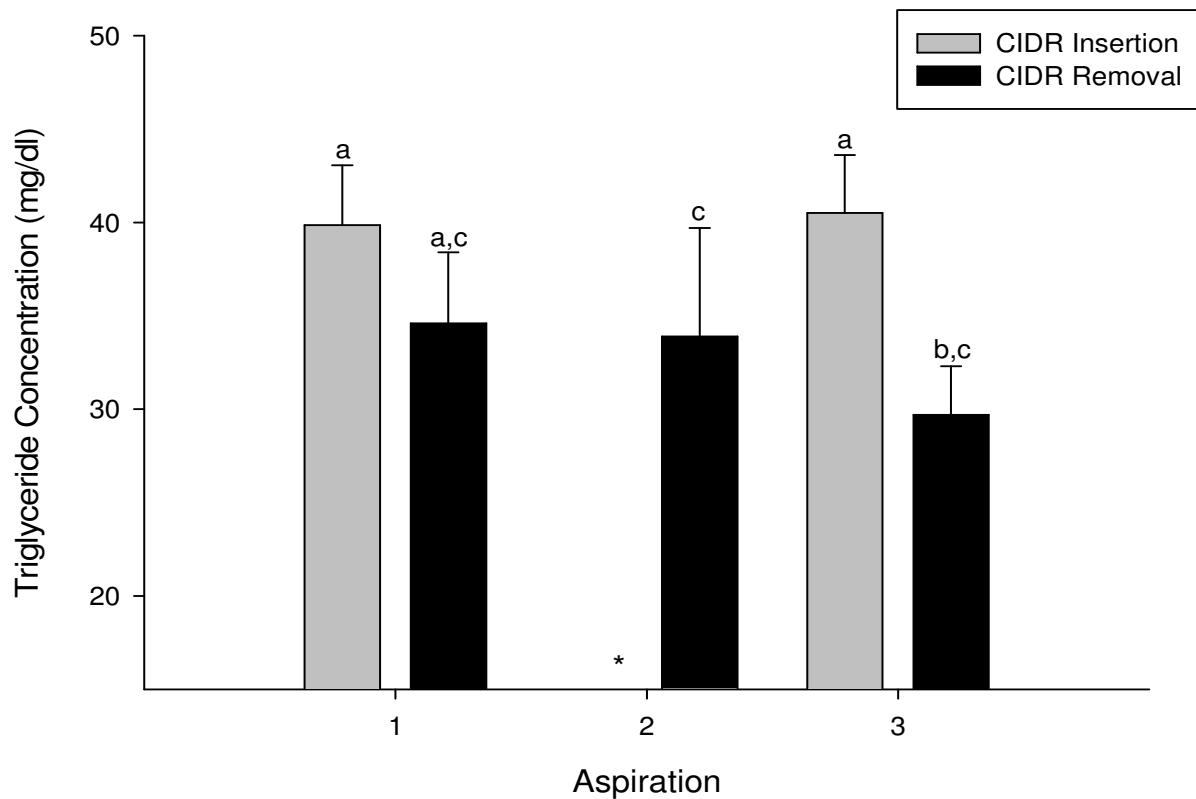


Figure 4.22. Mean (\pm SEM) triglyceride concentrations in Brahman donors at CIDR insertion and CIDR removal. ^{a,b}Means with different letters within aspirations are significantly different ($P < 0.05$). ^{c,d}Means with different letters between aspirations are significantly different ($P < 0.05$). *The serum at the time of CIDR insertion at aspiration 2 was mishandled and could not be analyzed for Brahman donor females.

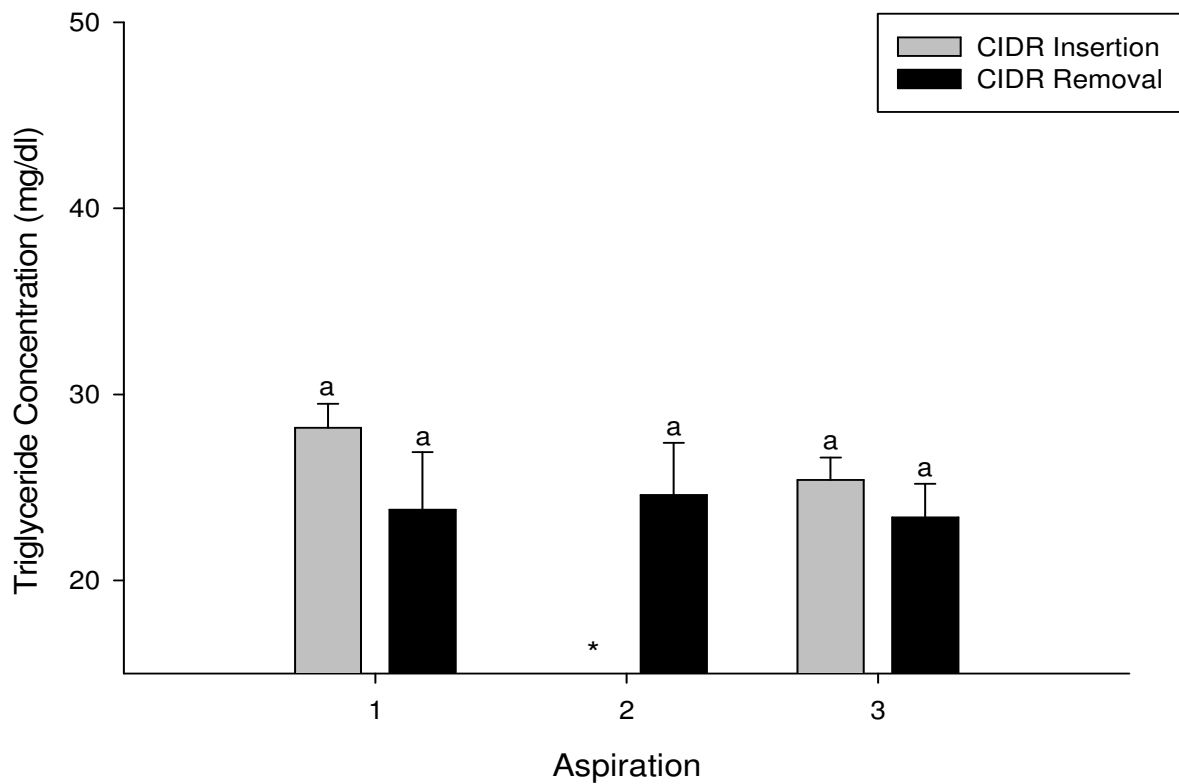


Figure 4.23. Mean (\pm SEM) triglyceride concentrations in Angus donors measured at CIDR insertion and CIDR removal grouped by aspiration. ^{a,b}Means with different letters within and between aspirations are significantly different ($P < 0.05$). *The serum at the time of CIDR insertion at aspiration 2 was mishandled and could not be analyzed for Angus donor females.

3. However, no significant difference was detected when comparing the overall mean circulating triglyceride concentrations in aspiration 2 between the Brahman and Angus donor females. No significant difference was detected when comparing the overall mean triglyceride levels for both breeds in each of the 3 aspirations (Figure 4.24).

The mean circulating triglyceride concentration in Brahman donors at CIDR insertion for the 3 aspirations was 38 ± 2.2 mg/dl. The mean triglyceride level for CIDR insertion was 27 ± 0.94 mg/dl. The mean circulating triglyceride concentrations over all 3 aspirations for Brahman and Angus donors at CIDR removal was 30.5 ± 2.5 and 22.5 ± 1.5 mg/dl. There was a significant decrease in triglyceride concentrations between both breed types at the time of CIDR insertion and CIDR removal (Figure 4.25).

Treatment Groups

When Replicates I and II were combined, all Brahman donor females were classified as treatment Group 1 and all the English breed females were classified as treatment Group 2 in this study. The mean number of follicles aspirated/donor and oocytes recovered/donor in the treatment group were 12 and 7 oocytes for Group 1 donor females and 18 follicles aspirated/donor and 13 oocytes/donor for Group 2 donor females.

Visual color scores of 1, 2 and 3 (light, medium and dark) were given to M-II oocytes prior to measurement in the sucrose buoyant density gradient. It was very difficult to detect color variation in the oocytes. The mean visual color scores of M-II stage Brahman and English breed oocytes were not significantly different ($P < 0.05$) (2.0 ± 0.01 and 1.9 ± 0.01 , respectively) (Table 4.1). In addition, the majority (98%) of Brahman and English M-II stage oocytes were determined to have a visual color score of 2. However, in the Brahman group, 6 M-II stage oocytes (2%) were given a visual color score of 3. In the English group, 4 M-II stage oocytes (2%) were classified as having a visual color score of 1.

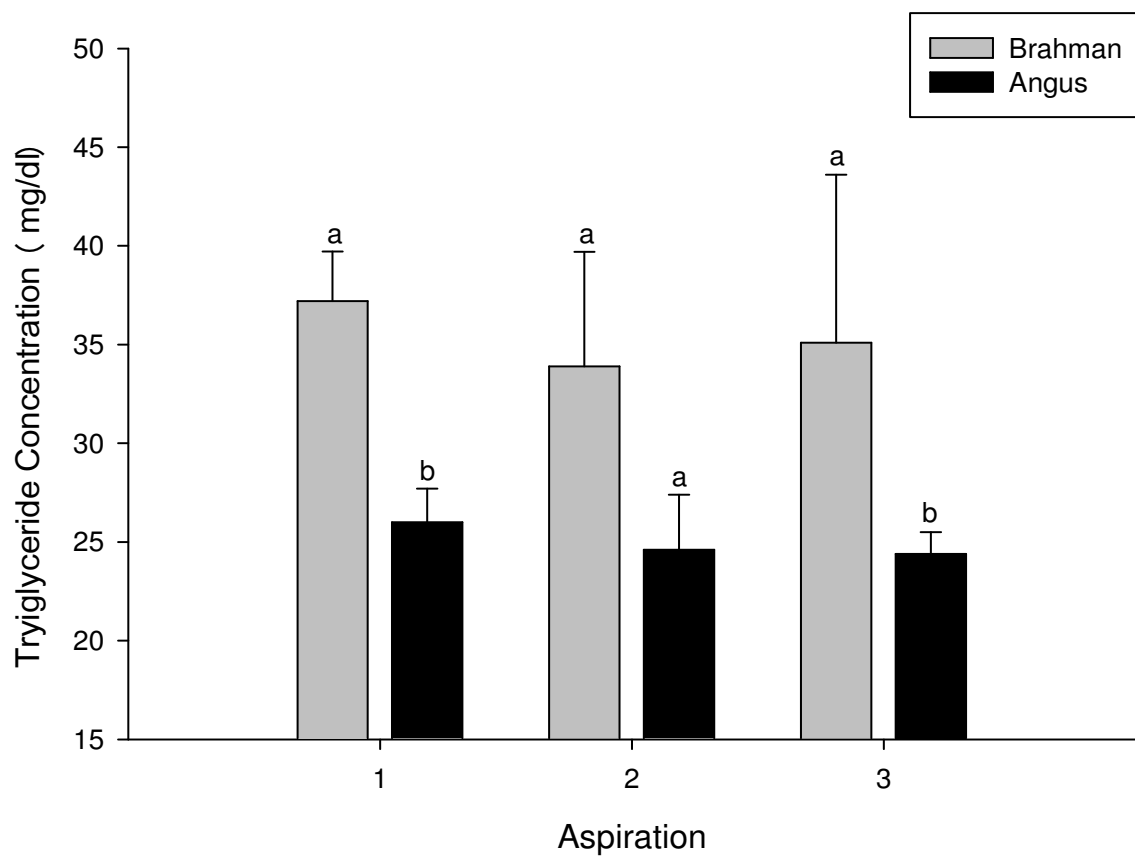


Figure 4.24. Overall mean (\pm SEM) triglyceride concentrations in Brahman and Angus donors when CIDR insertion and CIDR removal were combined by aspirations. ^{a, b}Means with different letters within aspiration are significantly different ($P < 0.05$).

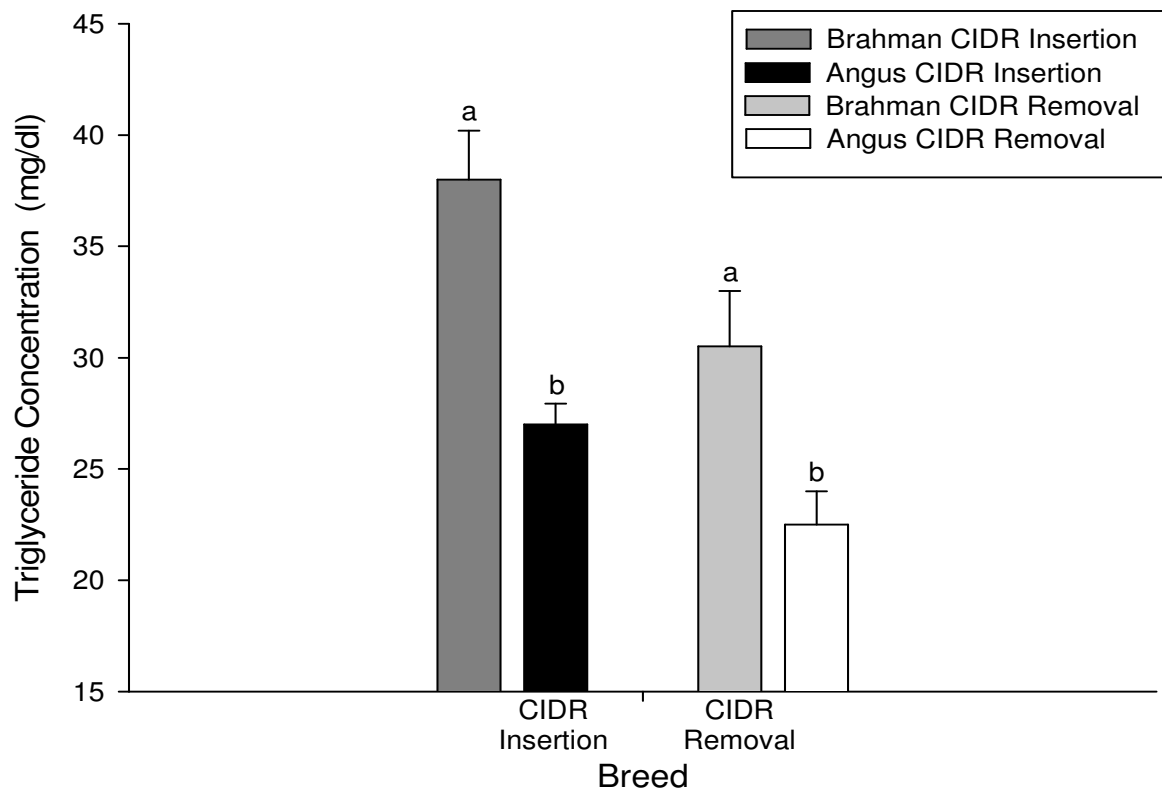


Figure 4.25. Mean (\pm SEM) triglyceride concentrations at the time of CIDR insertion and at CIDR removal (mean \pm SEM) in Brahman and Angus donors. ^{a,b}Means with different letters within breed types are significantly different ($P < 0.05$).

Table 4.1 Visual color score values of M-II stage Brahman and English breed donors oocytes

		Visual Color Score of Oocytes by Breed Type			
Group	Total M-II stage oocytes	Score 1 (%)	Score 2 (%)	Score 3 (%)	Mean (\pm SEM) color score
1 (Brahman)	242	0 (0)	236 (98)	6 (2)	2.0 \pm 0.1
2 (English)	170	4 (2)	166 (98)	0 (0)	1.9 \pm 0.1

Visual color scores of 1, 2 or 3 were given to Brahman and English breed M-II stage oocytes indicating light, medium or dark visual color, respectively.

The mean overall sucrose values of M-II oocytes for the Brahman (Group 1) and English breed (Group 2) donor females were $32.4\% \pm 0.10$ and $33.4\% \pm 0.13$, respectively. A significant increase ($P < 0.001$) of lipids was detected when the Brahman M-II stage oocytes were compared with English breed M-II oocytes (Figure 4.26).

Overall mean Nile Red fluorescence intensity in the Brahman and English breed oocytes were 173.4 ± 3.8 and 152 ± 4.4 Nile Red Units (NRU), respectively. A significantly higher ($P < 0.002$) fluorescence mean value was found when the data from the Brahman donors in Group 1 were compared with the mean NRU score for oocytes in the English breed donors in Group 2 (Figure 4.27).

The mean overall circulating cholesterol concentration when CIDR insertion and CIDR removal were combined in the Brahman donor females in Group 1 was 150.1 ± 4.9 compared with 109.6 ± 3.3 mg/dl for the English breed donor females. A significant decrease ($P < 0.001$) was noted between Group 1 and Group 2 donor females when CIDR insertion and CIDR removal were combined over aspirations (Figure 4.28). When the donors were grouped by CIDR insertion and CIDR removal, the mean cholesterol concentration for donor females in Group 1 were 148.3 ± 7.00 and 147.8 ± 5.67 mg/dl, respectively. The mean cholesterol concentrations for the Group 2 donor females at CIDR insertion was 108.3 ± 4.89 and 110.53 ± 4.45 mg/dl, respectively, at CIDR removal. A significant decrease in cholesterol concentration ($P < 0.05$) at CIDR insertion and CIDR removal was detected between Brahman (Group 1) and English (Group 2) donor females (Figure 4.29).

The overall mean circulating triglyceride concentrations for the Brahman and English breed donors were 33.2 ± 1.55 and 25.5 ± 1.23 mg/dl, respectively. The mean circulating triglyceride concentrations at CIDR insertion and at CIDR removal for Brahman donor females were 37.9 ± 1.86 and 28.4 ± 2.2 mg/dl. A significant decrease ($P < 0.05$) in triglyceride

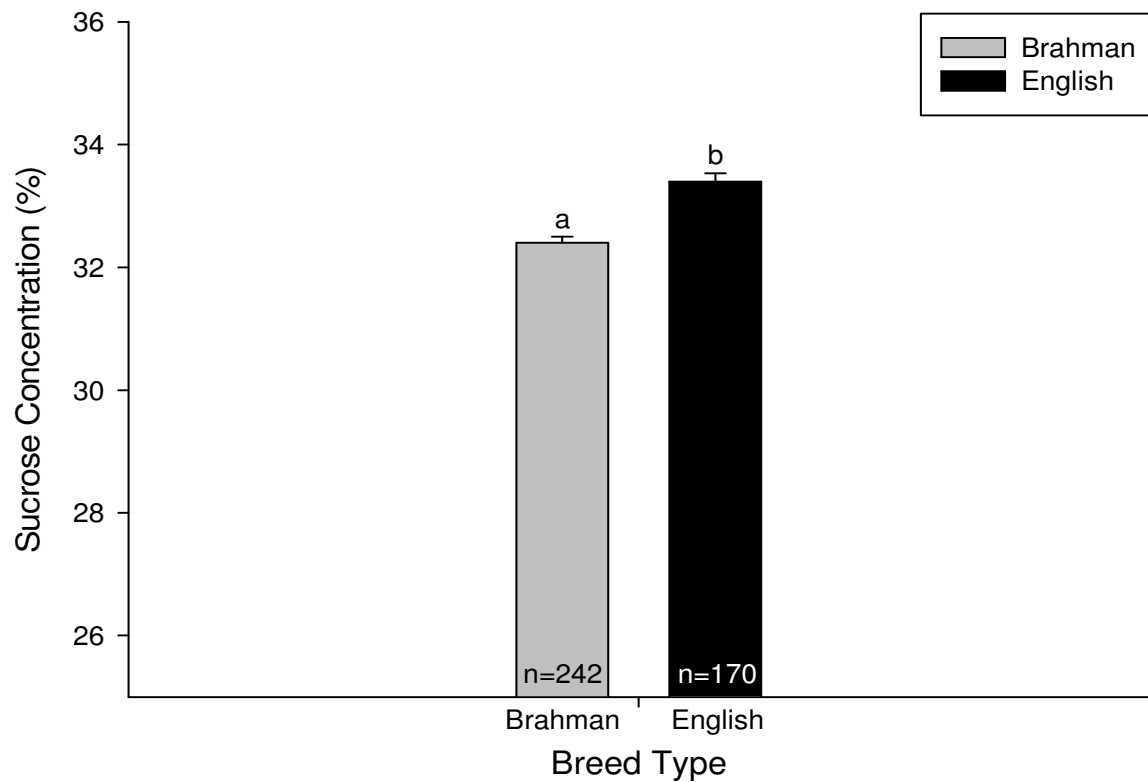


Figure 4.26. Mean (\pm SEM) sucrose buoyant density values for M-II stage oocytes from the Brahman and English breed type donors. ^{a,b}Means with different letters between breed type are significantly different ($P < 0.001$).

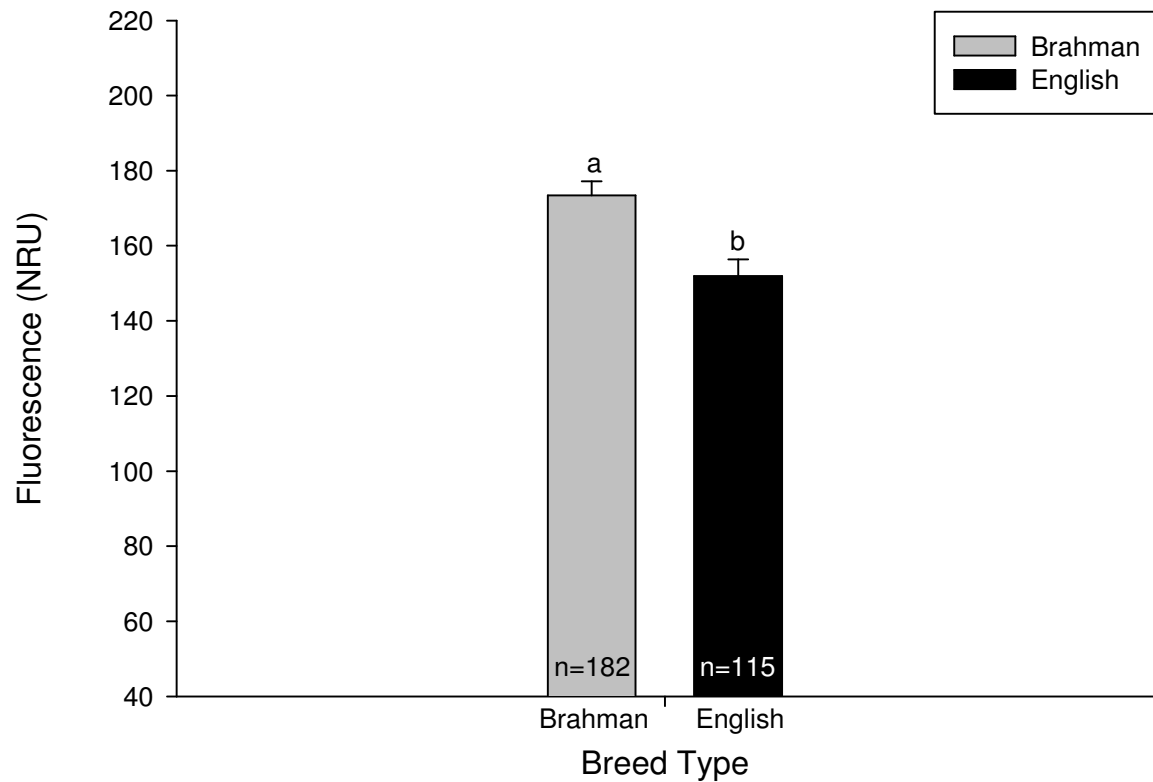


Figure 4.27. Mean (\pm SEM) Nile Red intensity (Nile Red units) of Brahman and English breed M-II stage oocytes after sucrose buoyant density measurements.
^{a,b}Means with different letters between breed types are significantly different ($P < 0.002$).

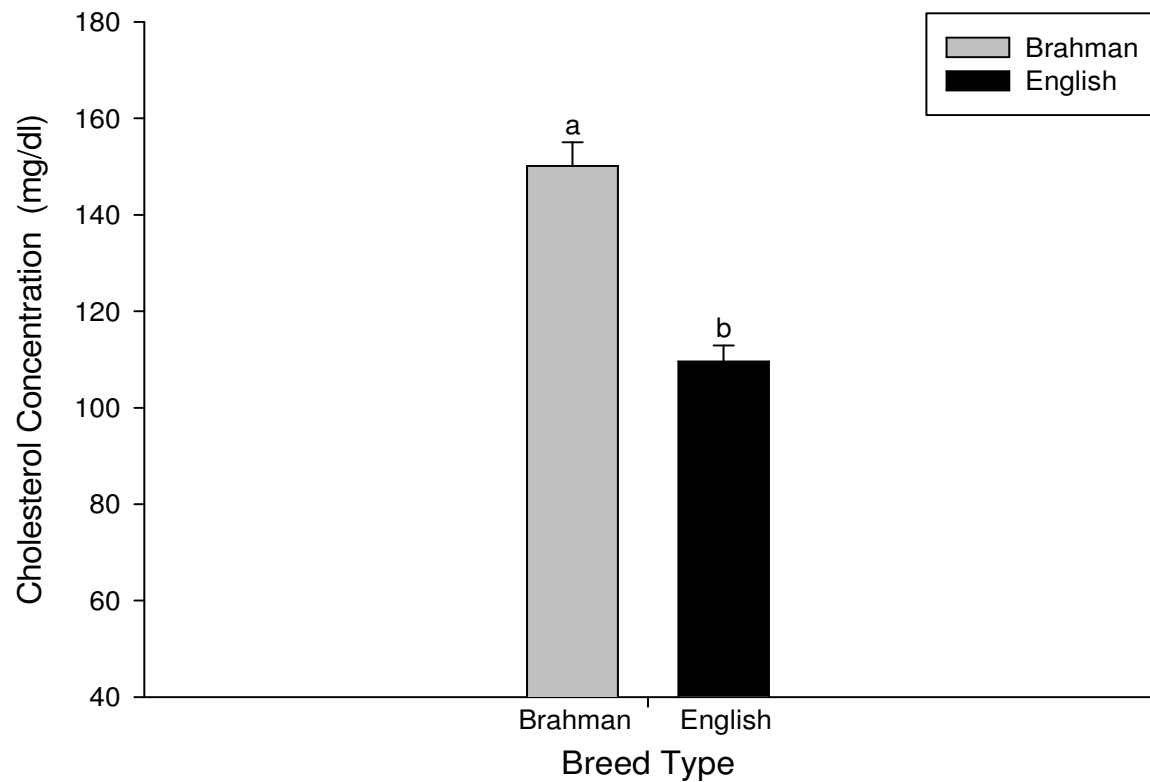


Figure 4.28. Mean (\pm SEM) cholesterol concentrations at CIDR insertion and CIDR removal in Brahman and English breed type donors. ^{a,b}Means with different letters between breed type are significantly different ($P < 0.001$).

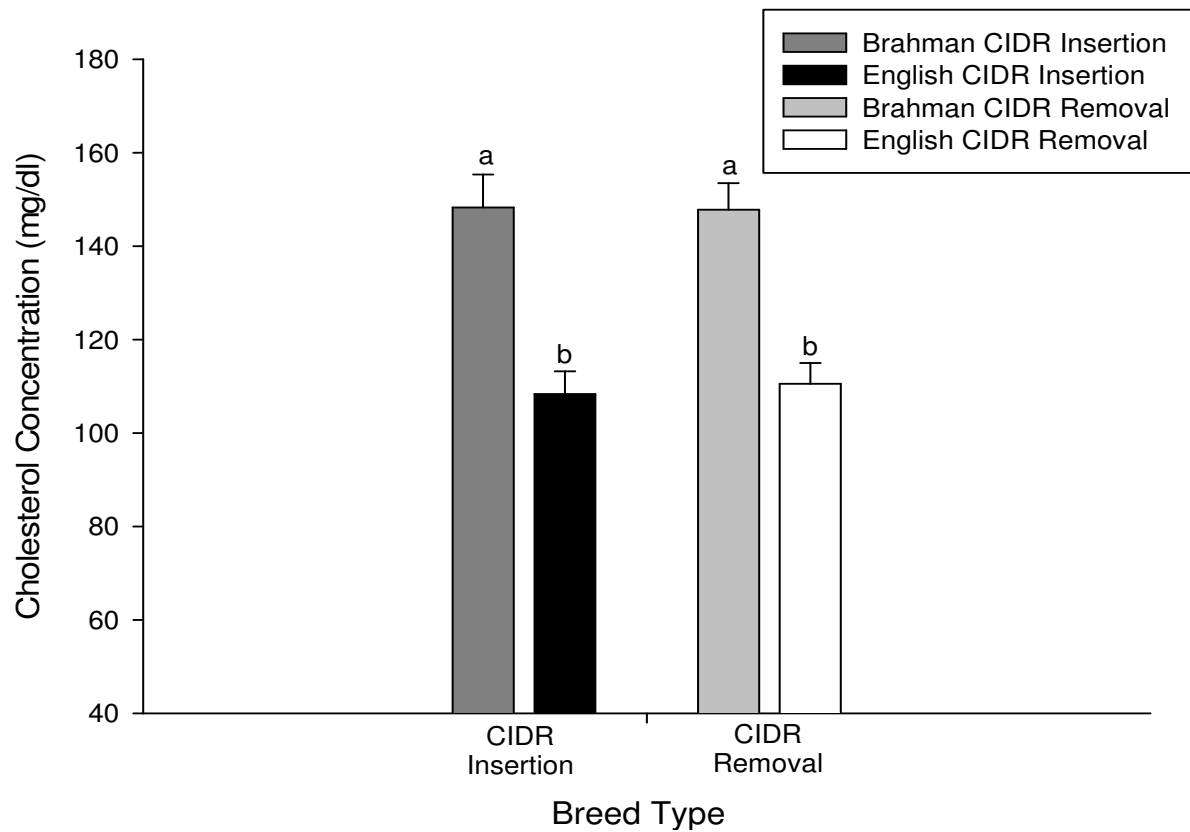


Figure 4.29. Mean (\pm SEM) cholesterol concentrations at CIDR insertion and CIDR removal in Brahman and English type donors. ^{a,b}Means with different letters within CIDR insertion and CIDR removal are significantly different ($P < 0.05$).

concentration was found when CIDR insertion and CIDR removal were combined over aspirations for Brahman donors (Group 1) when compared with English breed (Group 2) donor females (Figure 4.30). In the English breed donor females, circulating triglyceride concentration at CIDR insertion and CIDR removal were 29.71 ± 2.12 and 23.44 ± 1.32 mg/dl. There was a significant decrease ($P < 0.05$) found when Brahman donors (Group 1) were compared with English breed (Group 2) donor females for samples collected at the time of CIDR insertion and CIDR removal (Figure 4.31).

Body Condition Score (BCS) Groups

The Brahman and English breed donors from the breed type treatment groups (Group 1 and Group 2) were then combined based on body condition scores (scale of 1 to 9: 1 being thin and 9 being obese) for additional statistical analyses. Donors that had a high BCS (BCS ranged from 6 to 7.5) were then grouped and categorized as BCS Group 1 (English breed donor females in treatment Group 1 and Brahman breed donor females in treatment Group 2) and low BCS donors (BCS ranged from 4 to 5.5) were grouped and defined as BCS Group 2 (Brahman donors in treatment Group 1 and English donors in treatment Group 2).

The mean visual color scores of M-II stage oocytes for BCS Group 1 oocytes were 2.0 ± 0.01 and 1.9 ± 0.01 for the BCS Group 2 donor female oocytes. There was no significant difference ($P > 0.05$) noted when M-II oocytes visual color scores were compared between both BCS Groups. The majority of the BCS Group 1 and BCS Group 2 M-II stage oocytes were classified with a visual color score of 2 (97% and 98%, respectively). However, in the BCS Group 1, 6 M-II stage oocytes (3%) were assigned a visual color score of 3. In the BCS Group 2, 4 M-II stage oocytes (2%) were classified with a visual color score of 1 (Table 4.2).

The mean overall sucrose values for M-II oocytes for the BCS Group 1 and BCS

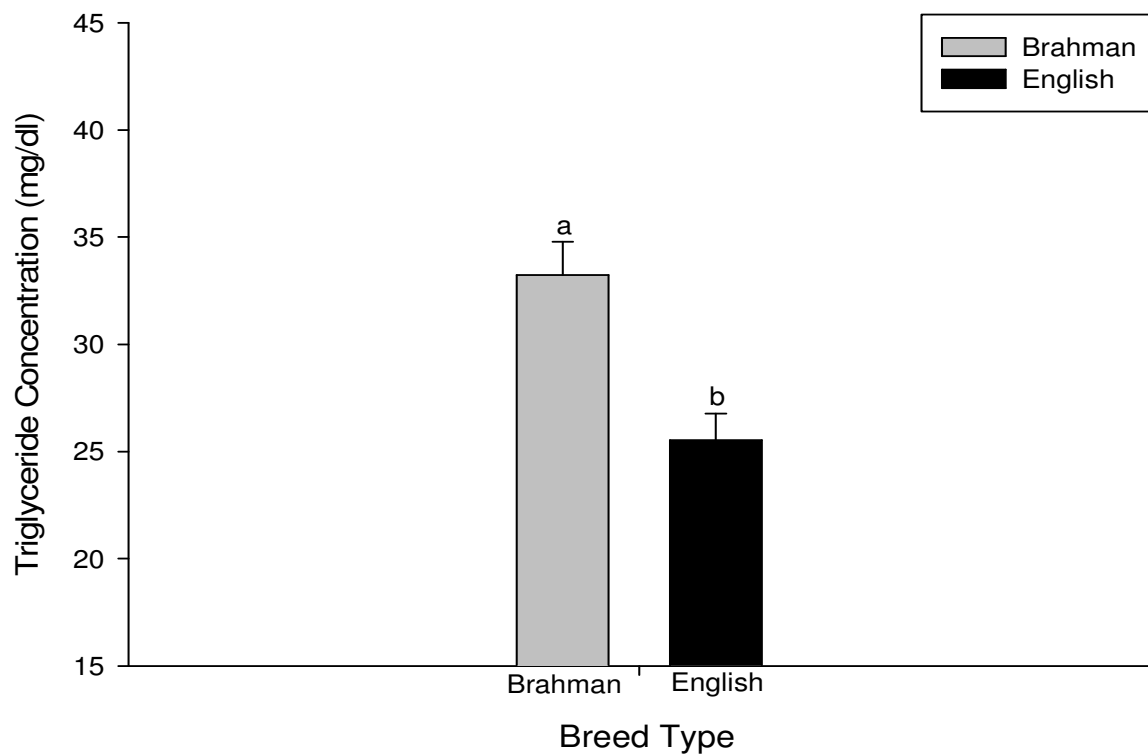


Figure 4.30. Mean (\pm SEM) triglyceride concentrations at CIDR insertion and CIDR removal in Brahman and English breed type donors. ^{a,b}Means with different letters between breed types are significantly different ($P < 0.05$).

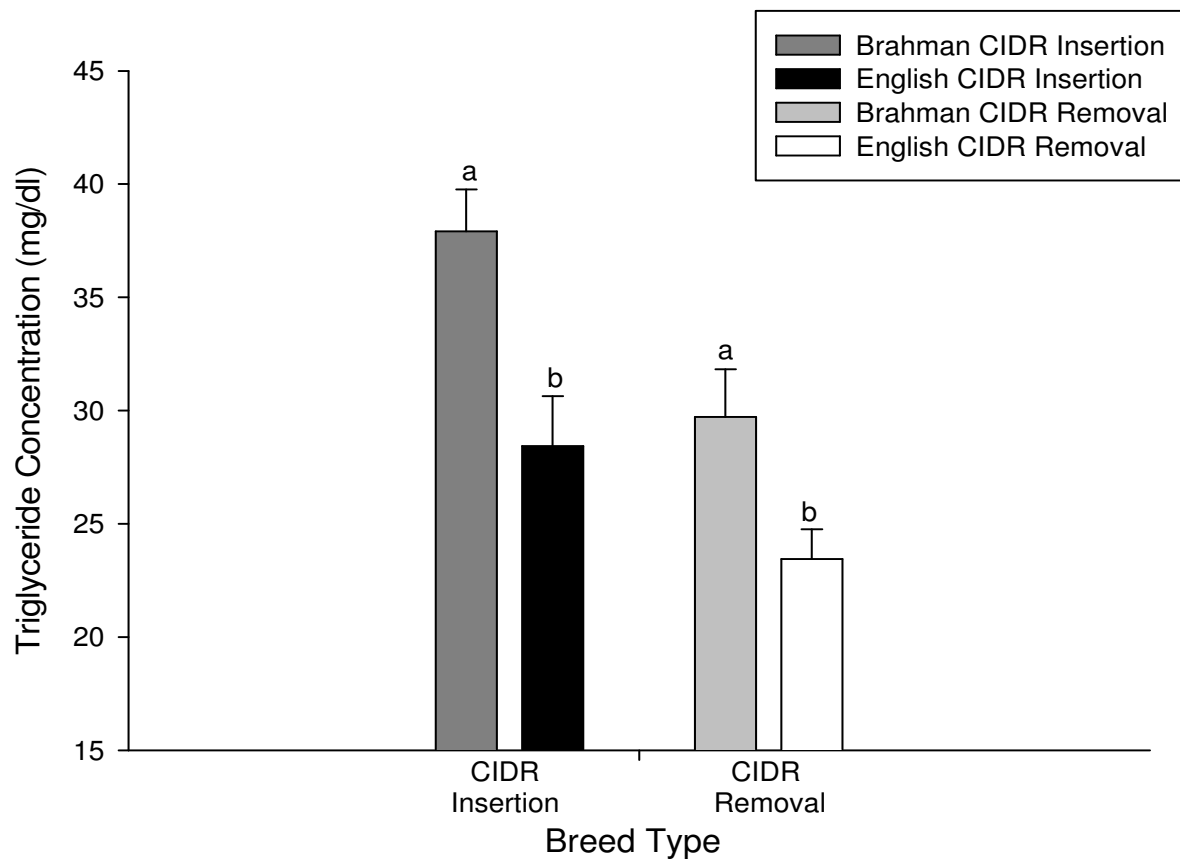


Figure 4.31. Mean (\pm SEM) triglyceride concentrations at CIDR insertion and CIDR removal in Brahman and English breed type donors. ^{a,b}Means with different letters within CIDR insertion and CIDR removal are significantly different ($P < 0.05$).

Table 4.2. Visual color score values of M-II stage BCS Group 1 and BCS Group 2 donor female oocytes

Group	Total M-II stage oocytes	Visual Color Score of Oocytes by Breed Type			
		Score 1 (%)	Score 2 (%)	Score 3 (%)	Mean (\pm SEM) color score
BCS 1	203	0 (0)	197 (97)	6 (3)	2.0 \pm 0.1
BCS 2	209	4 (2)	205 (98)	0 (0)	1.9 \pm 0.1

Visual color score of 1, 2 or 3 given to BCS Group 1 and BCS Group 2 M-II stage oocytes indicating light, medium or dark visual color, respectively.

Group 2 donors were $32\% \pm 0.10$ and $33\% \pm 0.12$, respectively. A significantly higher ($P < 0.001$) mean buoyant density sucrose value was noted when M-II stage oocytes from the BCS Group 1 were compared with BCS Group 2 donor females (Figure 4.32).

The mean overall Nile Red units of fluorescence intensity in the BCS Group 1 and BCS Group 2 were 181.4 ± 3.8 and 146.1 ± 4.1 NRU, respectively. A significant decrease in mean NRU ($P < 0.001$) was noted when the data from the BCS Group 1 were compared with the BCS Group 2 donor females (Figure 4.33).

The mean overall circulating cholesterol concentrations at CIDR insertion and CIDR removal for the BCS Group 1 and BCS Group 2 donor females were 124.7 ± 5.1 and 134.6 ± 6.2 mg/dl, respectively. No significant difference ($P > 0.05$) was found when CIDR insertion and CIDR removal were combined for both groups (Figure 4.34).

When the BCS groups were divided into CIDR insertion samples and CIDR removal samples, the mean circulating cholesterol concentrations for the BCS Group 1 were 136 ± 7.1 and 137 ± 6.8 mg/dl, respectively. The mean cholesterol concentrations for the BCS Group 2 donor females were 122.4 ± 7.4 and 119.2 ± 5.0 mg/dl at the time of CIDR insertion and CIDR removal. A significant increase ($P < 0.05$) in circulating cholesterol concentration was noted at the time of CIDR removal for BCS Group 1 when compared with CIDR removal for BCS Group 2. However, no significant difference ($P < 0.05$) of circulating cholesterol concentrations were noted at CIDR insertion between BCS Group 1 and BCS Group 2 (Figure 4.35).

The overall mean triglyceride concentrations for the BCS Group 1 donors were 25.9 ± 1.02 and 31.6 ± 2.4 mg/dl for the BCS Group 2. A significant increase in triglyceride concentration was noted when CIDR insertion and CIDR removal were combined for BCS Group 1 and compared with BCS Group 2 (Figure 4.36). The mean circulating triglyceride

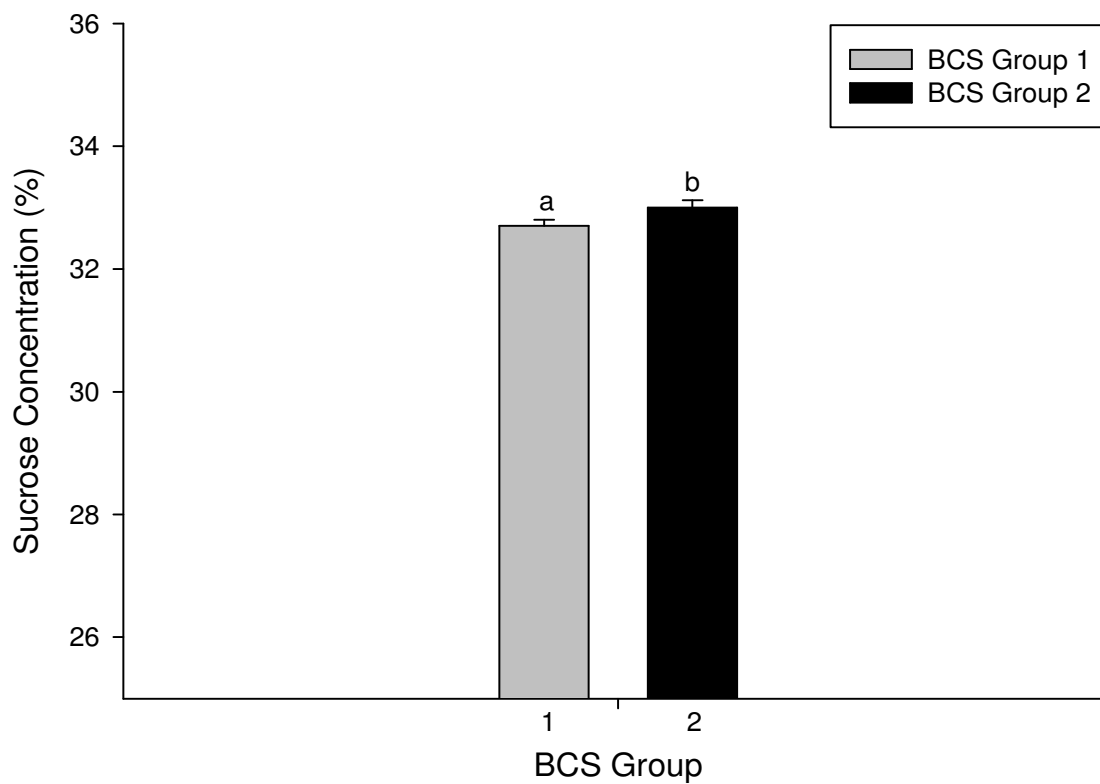


Figure 4.32. Mean (\pm SEM) sucrose buoyant density values for BCS Group 1 and BCS Group 2 M-II stage oocytes. BCS Group 1 = High BCS; BCS Group 2 = Low BCS. ^{a,b}Means with different letters between groups are significantly different ($P < 0.05$).

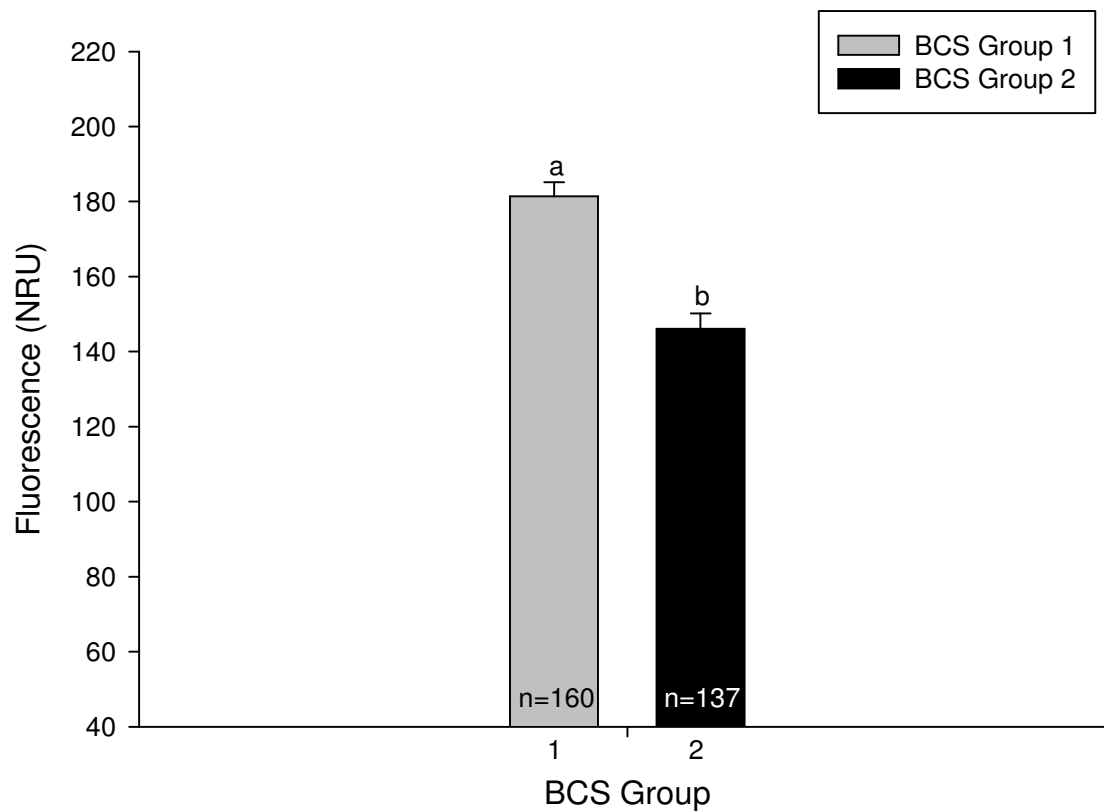


Figure 4.33. Mean (\pm SEM) Nile Red intensity (Nile Red units) of BCS Group 1 and BCS Group 2 M-II stage oocytes after buoyant density measurements. BCS Group 1 = High BCS; BCS Group 2 = Low BCS. ^{a,b}Means with different letters between groups are significantly different ($P < 0.05$).

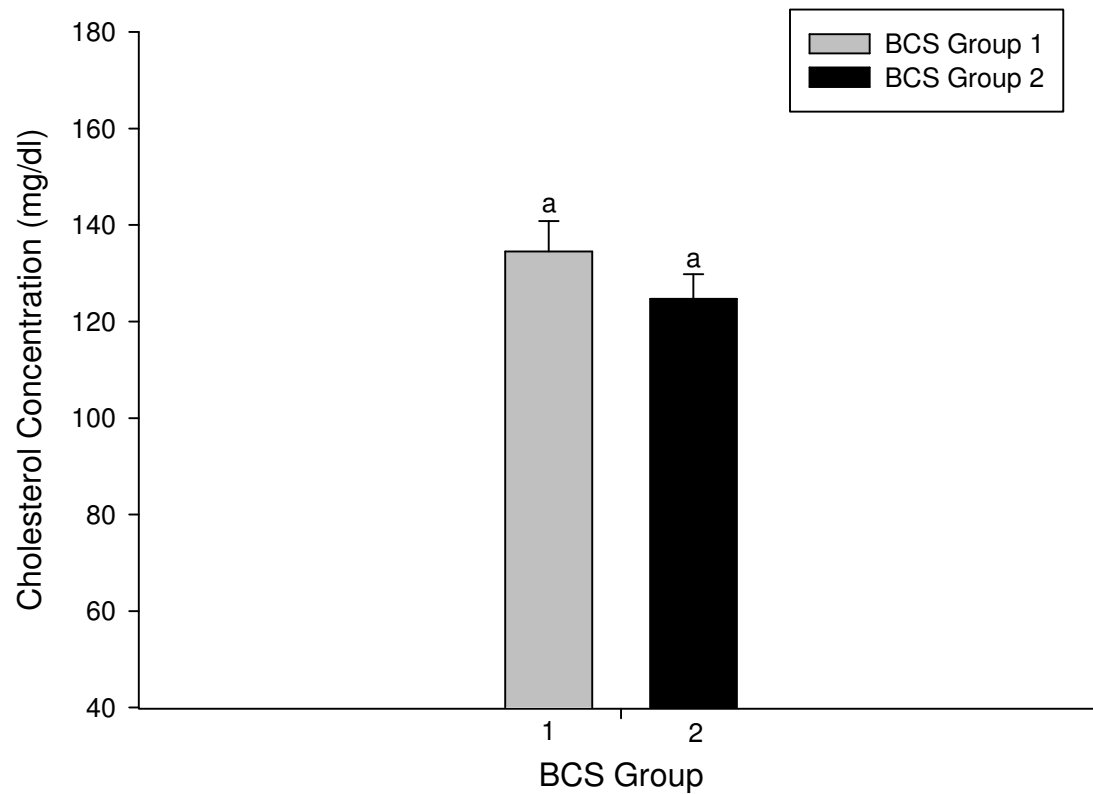


Figure 4.34. Mean (\pm SEM) cholesterol concentrations at the time of CIDR insertion and at CIDR removal for BCS Group 1 and BCS Group 2 donors. BCS Group 1 = High BCS; BCS Group 2 = Low BCS. ^{a,b}Means with different letters between groups are significantly different ($P < 0.05$).

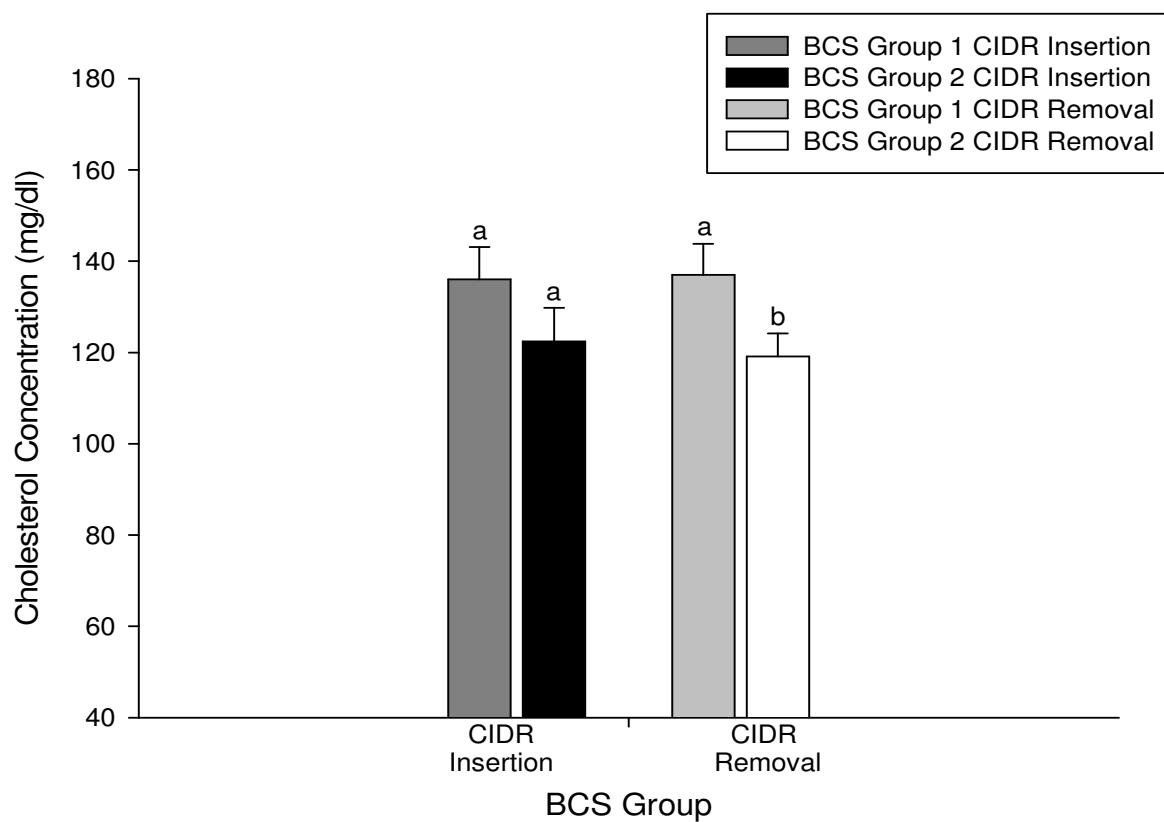


Figure 4.35. Mean (\pm SEM) cholesterol concentrations at CIDR insertion and CIDR removal of BCS Group 1 and BCS Group 2 donors. BCS Group 1 = High BCS; BCS Group 2 = Low BCS. ^{a,b}Means with different letters within CIDR insertion and CIDR removal are significantly different ($P < 0.05$).

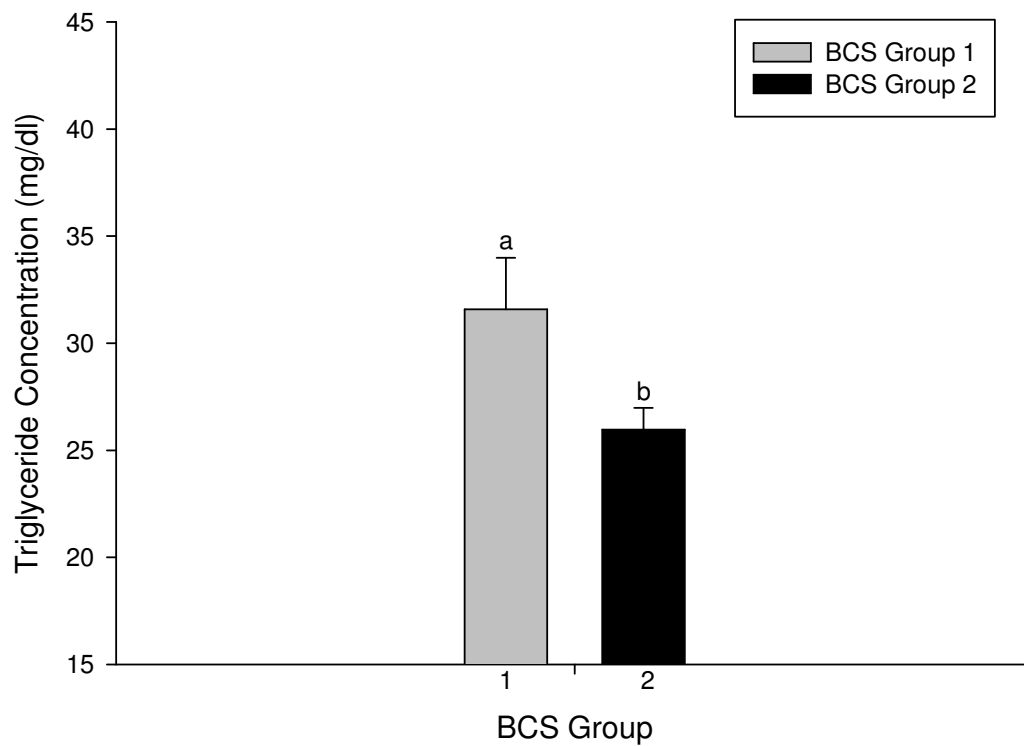


Figure 4.36. Mean (\pm SEM) triglyceride concentrations when CIDR insertion and CIDR removal were combined for BCS Group 1 and BCS Group 2 donors. BCS Group 1 = High BCS; BCS Group 2 = Low BCS. ^{a,b} Means with different letters between groups are significantly different ($P < 0.05$).

concentrations at CIDR insertion for BCS Group 1 were 37.7 ± 2.9 and 29.4 ± 1.3 mg/dl for BCS Group 2 donor females. The mean triglyceride concentrations at CIDR removal for BCS Group 1 were 30.2 ± 2.2 and 23.5 ± 1.3 mg/dl for BCS Group 2 donors. A significant decrease ($P < 0.05$) in circulating triglyceride concentration was noted for BCS Group 1 when compared with BCS Group 2 samples at CIDR insertion and CIDR removal (Figure 4.37).

DISCUSSION

In a replicates, lipid content of M-II stage oocytes from Brahman and English breed donor cows was compared with sucrose buoyant density gradient and with Nile Red staining. Data collected in this study indicate that Brahman M-II stage oocytes have a higher lipid content compared with English breed M-II stage oocytes, which may result in a lower tolerance to cryopreservation procedures and lower pregnancy rates. Evaluating serum concentrations, Brahman donors had higher circulating cholesterol and triglyceride levels when compared with the English breed donor females. In addition, higher circulating cholesterol and triglyceride concentrations in the donor females may directly influence lipid content found in oocytes. Although the cows were in different replicates of the same experiment, donor females were all fed the same diet for 25 days before and during the oocyte collection intervals. This reduced the chance that pre-treatment diet was the cause of the difference in oocyte lipid content and blood concentration of cholesterol and triglyceride found in this study (Adamiak et al., 2004; 2005).

At the time of collection, color and sucrose buoyant density gradient measurements density of the M-II stage oocytes was evaluated. Unfortunately, evaluating a visual color score of these M-II stage oocytes was very difficult under commercial conditions using a standard laboratory stereoscope. All the M-II stage oocytes were very similar in color among all groups of donors. However, a few oocytes from each group of donor females did have a

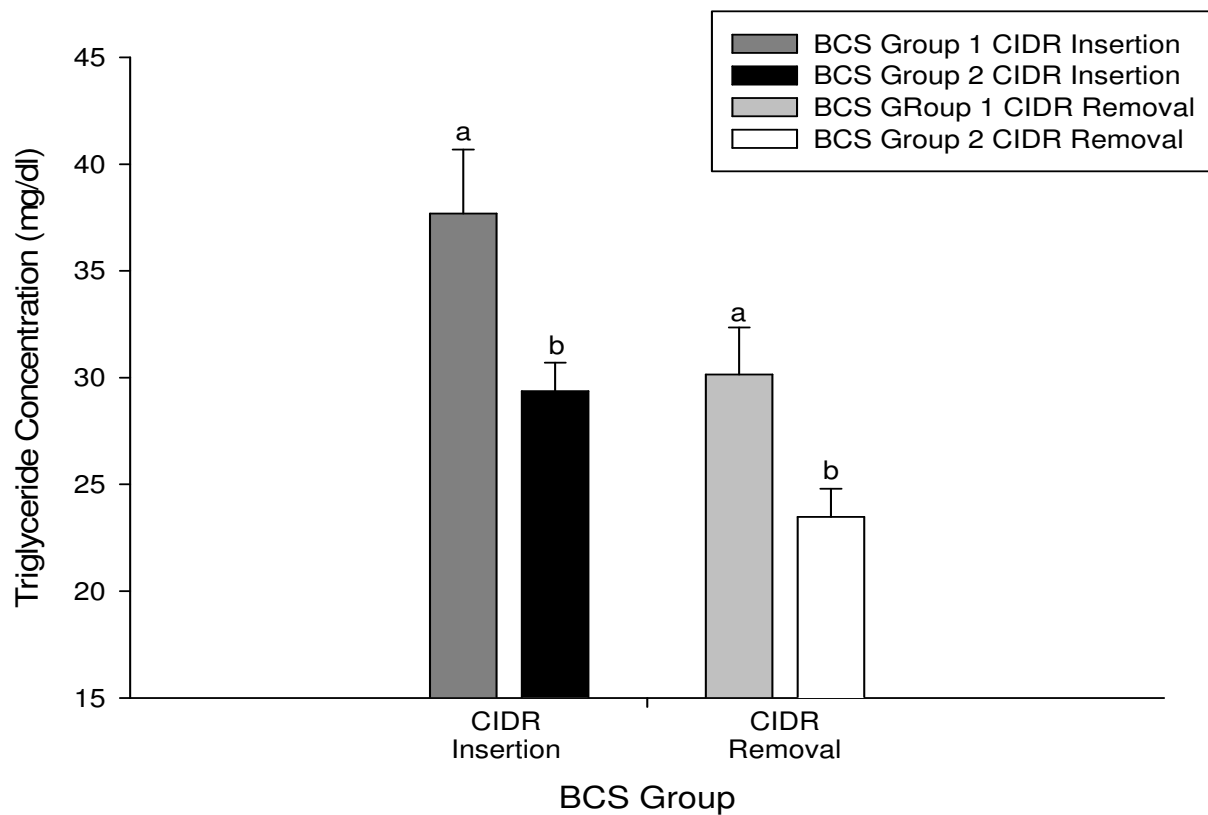


Figure 4.37. Mean (\pm SEM) triglyceride concentrations at CIDR insertion and CIDR removal for BCS Group 1 and BCS Group 2 donors. BCS Group 1 = High BCS; BCS Group 2 = Low BCS. ^{a,b}Means with different letters within CIDR insertion and CIDR removal are significantly different ($P < 0.05$).

visible difference in color density on appearance. This finding is in disagreement with the results reported by Hill et al. (1988) and Leory et al. (2004;2005), who reported a difference in visual color score of bovine embryos with different lipid content.

The main objective of using a sucrose buoyant density gradient was to measure lipid content of M-II stage oocytes using a simple indirect method based on settling points with known sucrose concentration layers. It was hoped that this method could be used to evaluate bovine oocytes under field conditions. The method of sucrose buoyant density gradient has previously been reported in similar experiments to measure the mass density of oocytes from copepods (Knutsen et al., 2001) and buoyancy of lipids in copepods (*Calanus finmarchicus*) (Visser et al., 1999). In preliminary experiments, we concluded that a sucrose buoyant density gradient is sensitive enough to detect differences in lipid content of both bovine and porcine M-II stage oocytes. This same approach will be useful to distinguish differences between M-II stage oocytes of different mammalian species.

Using a sucrose buoyant density gradient, we found a significantly higher oocyte lipid content when Brahman donors were compared with English breed donors when data were evaluated by breed types and grouped based on body condition scores (BCS). The results from Brahman donor females suggests that higher circulating triglyceride and cholesterol concentrations prior to oocyte collection were correlated with higher lipid content of M-II stage oocytes. When Brahman and English donor females were analyzed by BCS, the heavier BCS group had higher circulating triglyceride and cholesterol levels at CIDR insertion and CIDR removal (oocyte collection), which may be associated with the subsequent lipid content in M-II stage oocytes. The data obtained in this study support the hypothesis that Brahman embryos when compared with English breeds due indeed have a higher lipid

content, which may explain why Brahman embryos have a lower post-thaw pregnancy rate over that of English breed cattle.

Nile Red staining was performed to obtain a more precise quantification of lipid content in M-II stage oocytes, and the results re-enforced the findings obtained using a sucrose buoyant density gradient. Greenspan and Fowler (1985) used Nile Red as a fluorescent method to detect neutral intracellular lipids. Genicot et al. (2005) reported that porcine embryos had a higher fluorescence compared with bovine and murine oocytes when evaluated with Nile Red staining. Additionally, the use of Nile Red staining has also been used to evaluate lipid storage in copepods (Carman et al., 1991). The technique does not determine the type of lipids present just the approximate quantity present in the sample. Nile Red has been shown to be sensitive enough to compare lipid content between single oocytes of different species (Genicot et al., 2005). In this experiment, Nile Red was found to be sensitive when evaluating oocyte intracellular lipid content when compared with sucrose buoyant density measurements.

The Nile Red staining used in the experiment detected a significant difference in intracellular lipids among M-II stage oocytes between Brahman donors and English breed donors and when donor females were divided into high BCS and low BCS groups. The higher NRU indicated that there was a higher lipid content present in the M-II stage oocytes. Interestingly, high BCS and high cholesterol levels at CIDR insertion and CIDR removal in Brahman donor females was associated with higher lipid content of the M-II stage oocytes when compared with English breed group donors with low BCS grouping, which had low circulating cholesterol levels and lower oocyte lipid content. In addition, in the BCS groups, higher circulating triglyceride and higher circulating cholesterol levels correlated with the higher lipid content of M-II oocytes. The higher circulating cholesterol and triglyceride

concentrations noted in the donor females resulted in higher NRU in the M-II stage oocytes. The data obtained indicate that M-II oocytes from Brahman donors have higher intracellular lipids compared with the English breed types in this study.

Circulating cholesterol and triglyceride concentrations have previously been shown to be indicators of visual color levels in bovine embryos (Leroy et al., 2004). The color density of the porcine embryos has been associated with lipid droplet content (Abe et al., 1999; Abe et al., 2002). Leroy et al. (2005) reported that cattle with high circulating cholesterol and low circulating triglyceride levels produced embryos containing significantly darker embryos when compared with donors that had low circulating cholesterol levels and less dense embryos. Donor females with high circulating cholesterol levels at the time of embryo collection have been associated with low pregnancy rates after transferring morula stage embryos into synchronized cows (Hill et al., 1998).

In our experiment, circulating cholesterol and triglyceride concentrations were evaluated to determine if there was a correlation between these parameters and intracellular lipid content present in M-II stage oocytes. Leroy et al. (2004) have proposed serum cholesterol and triglyceride concentrations in donors might influence the lipid concentrations in the bovine embryos, which subsequently affected embryo freezability. In our study, both circulating cholesterol and triglyceride concentrations at CIDR insertion and CIDR removal (combined) were significantly different between the Brahman and English donor females. At CIDR insertion and CIDR removal, the Brahman group were high in both circulating cholesterol and triglyceride concentrations compared to the English breed group donor females. In addition, circulating cholesterol levels were higher in the BCS Group 1 when compared with the donors in the BCS Group 2. When comparing cholesterol and triglyceride levels at CIDR removal, the BCS Group 1 was significantly increased over that of the BCS

Group 2 donor females. Interestingly, when Brahman breed and English breed donors and BCS Group 1 and BCS Group 2 donor female M-II stage oocytes were evaluated for lipid content, high circulating cholesterol and high triglyceride concentration were correlated with sucrose buoyant density levels and Nile Red units (increased lipid content). These findings indicate that the evaluation of circulating cholesterol at the time of oocyte collection may be positively correlated to the amount of lipids present in M-II stage oocytes.

Body condition score, although subjective, is an indicator of nutritional status and the potential rebreeding performance in beef cattle after calving (Whitman, 1975, Wagner et al., 1988; Randel, 1990), and may also correlate to the oocyte lipid content prior to ovulation. In our experiment, when weight and BCS increased across aspirations of donors in both Groups 1 and 2, the intracellular lipid content of M-II oocytes also increased, as indicated by the Nile Red staining results. Moreover, sucrose buoyant density measurement also had a linear trend across aspiration replicates over all breed types. To date, the evaluation of BCS and body weight to determine the effect on lipid content in oocytes or embryos has not been reported. We hypothesize that these breed parameters may effect intracellular lipid content in M-II stage oocytes. It should not be over looked that breed types and BCS need to be taken into consideration when determining which embryos to cryopreserve when flushing donor cows.

The nutritional status of the donors prior to and during the experiment increased over aspiration replicates for both Brahman and English breed donor females, which was based on a slow but steady increase in cow body weight and BCS. Studies have reported a reduction in embryo quality from cattle donors fed elevated urea and protein levels (Elrod et al., 1993; Dawuda et al., 2002). However, the effect of feed intake on oocyte quality is likely dependent on the body condition of the cow, with the high level of feeding being beneficial to

oocytes from low body condition animals but less beneficial to oocytes from animals of moderately high body condition (Adamiak et al., 2005).

Diets high in fat can have profound effects on reproduction in many species. Dietary fat has been hypothesized to increase steroidogenesis favorably to improved fertility, increase ovarian follicular development through manipulation of insulin concentrations and stimulate or inhibit the release of $\text{PGF}_{2\alpha}$, which influences the persistence of the CL (Grummer et al., 1991; Staples et al., 1998).

Lammoglia et al. (2000) reported that feeding cows supplemental dietary fats can increase serum progesterone by increasing lipid content in the small and large luteal cells. In addition, serum and follicular fluid cholesterol concentrations also increase in these cattle. The unsaturated fatty acids may have an effect on the environment surrounding the oocyte, which provides essential nutrients for the oocyte or embryo survival post-ovulation (Biby et al., 2006).

During the preiovulatory period, oocytes undergo nuclear and cytoplasmic maturation. During this process, fatty acids are required for cell structure, function and metabolism, which may explain the increased cholesterol detected in the follicular fluid. However, the amount of information on the effect of dietary fat on intracellular lipid content in cattle oocytes and embryos is limited.

The reduction in embryo quality may be altered at early folliculogenesis and possibly at the follicle cohort level rather than later when embryos are developing in the reproductive tract of the female (Leory et al., 2004). Findings of Sata et al. (1999) and Kim et al. (2001) support the hypothesis that bovine embryo quality is affected prior to follicular growth based on experiments where oocytes were cultured in different media which showed oocytes accumulated lipids as they developed.

In conclusion, the results from sucrose buoyant density and Nile Red staining indicate that nonlactating Brahman donor females have a higher lipid content in their M-II stage oocytes compared with nonlactating English breed females. In this experiment, using breed type, BCS, circulating cholesterol and triglyceride concentrations may help recognize donor females with high lipid content in oocytes. This should ultimately help improve embryo selection for cryopreservation and improve pregnancy rates from cryopreserved embryos.

The ultimate goal is to develop a simple method to identify Brahman donor cows that would have the lowest oocyte lipids and thus, improve pregnancy rates from cryopreserved embryos. At this stage, one should not overlook the use of a blood sample collected at the time of CIDR insertion (start of the estrus synchronization protocol) and a CIDR removal to monitor cholesterol and triglyceride concentrations, as an indicator of oocyte lipid content. In addition, further research is needed to determine why Brahman cattle have more lipids present in their oocytes and how they accumulate these lipids into the oocytes.

CHAPTER V

SUMMARY AND CONCLUSIONS

The primary objectives of this series of experiments was to determine intracellular lipid content in mature oocytes of Brahman and English cattle using a sucrose buoyant density gradient and Nile Red staining. Information regarding the amount of intracellular lipids in oocytes between breeds is limited. The determination of the lipids present in oocytes of different breeds will allow improvements in cryopreservation and may lead to alternative feeding protocols for donors.

In the first experiment, intracellular lipids present in M-II oocytes from different species were determined to be different with a sucrose buoyant density gradient and Nile Red staining. Results showed, that using a sucrose step gradient and using Nile Red stain can reliably distinguish the lipid content present in oocytes from different species. Maximum settling time for M-II oocytes was observed at 1 hour. Furthermore, the maximum time required for oocyte incubation in Nile Red was 24 hours. We can conclude that M-II porcine oocytes have high intracellular lipid content when compared with bovine and murine oocytes.

In the second experiment, Brahman and English (Hereford and Angus) M-II oocytes were evaluated to determine if there was a difference in lipid content using a buoyant density gradient and Nile Red staining. It was determined that M-II oocytes from Brahman cattle have a higher lipid content when compared with the English breed types. Furthermore, using Nile Red stain to determine lipid content in oocytes is a more precise measurement than using buoyant density gradient.

Additionally, in the second experiment, breed parameters and serum levels were obtained on Brahman and English breed donors to determine their effect on intracellular lipid content in M-II oocytes. Results indicate that body condition score and cholesterol level had

were significantly higher when intracellular lipid content of oocytes were greater using sucrose buoyant density and Nile Red for Brahman and English donors.

In conclusion, data obtained from these experiments support the hypothesis that Brahman cattle have higher lipid content in oocytes compared with English breed types. This is the first report to evaluate intracellular lipid content in M-II oocytes from *Bos indicus* and *Bos taurus* breeds.

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APPENDIX: RESEARCH PROTOCOLS

A

BOVINE OOCYTE ASPIRATION POTOCOL

Materials:

Oocyte Maturation Medium (OCM)
L-Glutamine
BSS + heparin
Penicillin/streptomycin
Saline solution (0.9%)
Petri dishes
Bench top paper
400 ml beaker
Scalpel handle
Scalpel blades (sizes #11 and #20)
Hemostat
Mouth pipette

Preparations:

- A. Oocyte Collection Medium (OCM) + Supplements
 - 1. Prepare OCM + Supplements by adding the following to one liter of OCM:

BSS + heparin (stock)	20 ml
Penicillin (Gibco 15140-122)	10 ml
L-Glutamine (Gibco 25030-081)	10 ml
 - 2. Place OCM supplements at room temperature at least two hours before arrival of ovaries.
- B. Set Up for Collection (1 station per person)
 - 1. Cover bench top with paper.
 - 2. Add the following to the bench top:
 - 50 ml test tube
 - 20.5 gauge needles
 - 10 ml embryo friendly syringe (Norm-Ject)
 - Hemostats
- D. Saline brought to room temperature to wash ovaries.

Procedures:

- A. Clean the ovaries with saline solution
- B. Aspiration
 - 1. Add 25 ml OCM to each test tube.
 - 2. Attach needle to syringe.
 - 3. Insert needle from one side and puncture follicles from the bottom in the size of 3 to 10 mm. Try not to pull needle out of ovary. Both follicular fluid and blood in the collection medium could result in clotting of the medium, thereby rendering it impossible to retrieve oocytes. To prevent clotting of the medium, avoid aspirating large follicles (>10 mm) and corpora lutea.

4. Dispense oocytes and follicular fluid into 50 ml test tube that is filled with 25 ml OCM.

Searching for oocytes:

1. Once a group of ovaries have been processed, fill the beaker with OCM and incubate at room temperature for five minutes to allow oocytes to settle.
2. Bathe the outside of the test tube with ETOH and transfer the tube to the hood and allow oocytes to settle again for a few minutes.
3. Using aseptic technique, slowly aspirate OCM from the top of the beaker down to 50 ml. Be careful to not disturb the oocytes on the bottom of the test tube. Stop immediately if this should occur, and allow the oocytes to settle again. Fill the tube again with OCM and let settle for another 5 minutes, and slowly aspirate down to 50 ml.
4. Transfer remaining media, with oocytes, to two grid plates. Wash the beaker with about 20 ml of OCM and add to the grid plates.
5. Collect cumulus oocyte complexes (COC) as fast as possible using a mouth pipette. Place retrieved COC's into the first Petri dish containing OCM for further washing.
6. Transfer COC's from first dish to the next leaving all debris behind (repeat twice to assure that oocytes are clean of debris).

B

PORCINE *IN VITRO* MATURATION MEDIUM

Medium:

1. 8.9 ml TCM-199 medium
2. 0.1 ml penicillin/streptomycin
3. 1.0 ml porcine follicular fluid (final concentration 10%)
4. 1.0 mg cystein ($\text{HSCH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H}$), (Final concentration 0.1 mg/ml)
5. 10 μl EGF stock (10 $\mu\text{g/ml}$; final concentration 10 ng/ml)

Sterile filter:

Make Nunc 4-well maturation dish with 500 μl medium in each well

Add into each well:

1. 5 μl bFSH stock (50 IU/ml; final concentration 0.5 IU/ml)
2. 5 μl bLH stock (50 IU/ml; final concentration 0.5 IU/ml)

Procedure:

Porcine ovaries will be collected at a local abattoir. Oocytes will be aspirated from follicles of 3 to 8 mm in diameter using a 20 ml embryo friendly (Norm-Ject) syringe and a 20.5-gauge needle and transferred to maturation medium: tissue culture medium TCM-199 (8.9 ml) supplemented with 10% (v/v) fetal calf serum, 0.1 ml penicillin/streptomycin, 0.1 mg/ml cystein, 10 μl EGF, 5 μl FSH and LH (0.5 IU/ml). Oocytes will be cultured at 38°C in 5% CO_2 in humidified air. After 42 hours of maturation, oocytes will be denuded by vortexing at high speeds for 3 minutes with Hypes 1 ml + hyaluronidase 0.001 g in a 15 ml conical tube.

C

BOVINE *IN VITRO* MATURATION MEDIUM

Medium:

1. 8.635 ml TCM-199 medium
2. 0.020 ml bFSH stock
3. 0.125 ml bLH stock
4. 1.000 ml FBS
5. 0.010 ml Gentamicin
6. 0.010 ml E₂ stock

Hormone stock:

bFSH: 1 vial contains 490 IU (~35 mg)

Reconstitute with 14 ml TCM to give 2.5 mg/ml stock (35 IU/ml)

Aliquot in 50 µl portions and store @ -80°C

Add 0.020 ml stock to 10 ml maturation medium to give 5 µg/ml (0.7 IU/ml)

bLH: 1 vial contains 30,000 IU (~8 mg)

Reconstitute with 10 ml TCM to give a 0.8 mg/ml stock (3000 IU/ml)

Aliquot in 200 µl portions and store @ -80°C

Add 0.125 ml to 10 ml maturation medium to give 10 µg/ml (37.5 IU/ml)

E₂: Add 10 mg estradiol to 10 ml 95% EtOH to give 1 mg/ml stock store @ -80°C

1. Add 8.635 ml TCM, 0.020 ml FSH, 0.125 ml LH stock, 1 ml FBS and 0.010 ml gentamicin to a 15 ml tube. Sterile filter.
2. Add 0.010 ml estradiol stock
3. Prepare 35 µl maturation droplets of filtered medium under oil and equilibrate in incubator (5% CO₂) for at least 3 hours
4. Prepare 75 µl wash droplets as above

Procedure:

Oocytes will be aspirated from follicles of 3 to 8 mm in diameter using a 20 ml embryo friendly syringe (Norm-Ject) and a 20.5-gauge needle and transferred to maturation medium: tissue culture medium TCM-199 supplemented with 10% (v/v) fetal bovine serum, 100 µg/ml sodium pyruvate, gonadotropins (0.05 IU/ml FSH and 0.05 IU/ml LH). Oocytes will be cultured at 38°C in 5% CO₂ in humidified air. After 20 hours of maturation, oocytes will be denuded by vortexing at high speeds for 3 minutes with Hypes 1 ml + hyaluronidase 0.001 g in a 15 ml conical tube.

D**SUCROSE BUOYANT DENSITY GRADIENT****Medium:**

1. Dulbecco's PBS	Sucrose (C ₁₂ H ₂₂ O ₁₁) (g)	Final Concentration (%)
25 ml	4.00 g	16%
25 ml	4.50 g	20%
25 ml	5.50 g	22%
25 ml	5.75 g	23%
25 ml	6.00 g	24%
25 ml	6.25 g	25%
25 ml	6.50 g	26%
25 ml	6.75 g	27%
25 ml	7.00 g	28%
25 ml	7.25 g	29%
25 ml	7.50 g	30%
25 ml	7.75 g	31%
25 ml	8.00 g	32%
25 ml	8.25 g	33%
25 ml	8.50 g	34%
25 ml	8.75 g	35%

Procedures:

Sucrose buoyant density step gradient was prepared individually in a 50 ml conical test tube. Sucrose was measured in grams using weighing paper with an analytical balance, CP Series, Sartorius. Before each measurement, scale was reset to 0 g. Sucrose was deposited into the 50 ml sterile centrifuge test tube then Dulbecco's PBS was added until the meniscus reached the 25 ml mark on tube. Medium was stirred until sucrose was dissolved. Each level was carefully dispensed in descending order, starting with 30%, as to not disrupt the levels.

E**FIXATIVE MEDIUM****Medium:**

1. 108 µl Formaldehyde (16%)
2. 250 µl Glutaraldehyde (50%)
3. 1642 µl Dulbecco's PBS

Preparing Fixative Medium:

1. Medium needs to be made on the day of fixing samples
2. Dispense Dulbecco's PBS, 0.8 % formaldehyde and 6.25% glutaraldehyde into 15 ml conical tube wrapped with tin foil. Medium is light sensitive.
3. Store at 4 °C on the day on mixing and while using medium

F

NILE RED STAIN

Medium:

1. 50 µl of Nile Red stock solution,
2. 4950 µl of Sodium Chloride (NaCl) (0.9%)
3. 5 mg of Polyvinyl-Pyrrolidone (C₆H₉NO) (PVP)

Nile Red stock:

1. Nile Red stock solution (1 µg/ml) was prepared by dilution in DMSO and stored at room temperature in the dark.

Preparing Nile Red stain:

1. Prepare stain in 25 ml conical tube wrapped in tin foil. Solution is light sensitive.
2. Centrifuge at 2500g for 10 minutes then filter solution using a 0.25 µm filter attached to a 25 ml embryo friendly (Norm-Ject) syringe.
3. Droplets for oocytes will contain 30 µl each and mature oocytes will be incubated at room temperature for 24 hours.

G

SUPERSTIMULATION TREATMENTS (BRAHMAN)

Procedure:

	<u>Dose</u>	<u>Drug</u>	<u>Day</u>	<u>AM/PM</u>
CIDR Insertion			0	AM
Combo (25mg + 1.25 mg/ml)	2.0 ml	P ₄ +E ₂	0	AM
FSH (20 mg/ml)	2.7 ml	FSH-V	4	AM/PM
FSH (20 mg/ml)	2.2 ml	FSH-V	5	AM/PM
FSH (20 mg/ml)	1.7 ml	FSH-V	6	AM/PM
			7	
CIDR Removal			8	AM
Oocyte Retrieval			8	AM

SUPERSTIMULATION TREATMENTS (HEFEFORD)

Procedure:

	<u>Dose</u>	<u>Drug</u>	<u>Day</u>	<u>AM/PM</u>
CIDR Insertion			0	AM
Combo (25mg + 1.25 mg/ml)	2.0 ml	P ₄ +E ₂	0	AM
FSH (20 mg/ml)	2.2 ml	FSH-V	4	AM/PM
FSH (20 mg/ml)	1.7 ml	FSH-V	5	AM/PM
FSH (20 mg/ml)	1.2 ml	FSH-V	6	AM/PM
			7	
CIDR Removal			8	AM
Oocyte Retrieval			8	AM

H

SUPERSTIMULATION TREATMENTS (BRAHMAN)

Procedure:

	<u>Dose</u>	<u>Drug</u>	<u>Day</u>	<u>AM/PM</u>
CIDR Insertion			0	AM
Combo (25mg + 1.25 mg/ml)	2.0 ml	P ₄ +E ₂	0	AM
FSH (20 mg/ml)	2.3 ml	FSH-V	4	AM/PM
FSH (20 mg/ml)	2.0 ml	FSH-V	5	AM/PM
FSH (20 mg/ml)	1.5 ml	FSH-V	6	AM/PM
			7	
CIDR Removal			8	AM
Oocyte Retrieval			8	AM

SUPERSTIMULATION TREATMENTS (ANGUS)

Procedure:

	<u>Dose</u>	<u>Drug</u>	<u>Day</u>	<u>AM/PM</u>
CIDR Insertion			0	AM
Combo (25mg + 1.25 mg/ml)	2.0 ml	P ₄ +E ₂	0	AM
FSH (20 mg/ml)	2.6 ml	FSH-V	4	AM/PM
FSH (20 mg/ml)	2.4 ml	FSH-V	5	AM/PM
FSH (20 mg/ml)	2.0 ml	FSH-V	6	AM/PM
			7	
CIDR Removal			8	AM
Oocyte Retrieval			8	AM

IN VITRO MATURATION MEDIA
(Ovagenix, Bryan, TX)

Medium:

1. 4400 µl TCM 199 w/Earle's salts
2. 500 µl Characterized fetal bovine serum
3. 25 µl bFSH
4. 25 µl bLH
5. 50 µl Penicillin streptomycin

Equipment:

1. 5 ml Disposable pipette and bulb
2. P-20 and P-200 Eppendorf pipette with tips
3. 14 ml Snap cap tubes
4. 10 ml Norm-Ject syringe
5. 0.2 µm Acrodisc syringe filter
6. 0.65 ml Micro-centrifuge tubes
7. -20°C Non-Frost free freezer

Gonadotropin stock solutions:

bFSH

1. Dilute (1) 50-unit vial of bFSH with 25 ml of TCM199
2. Aliquot 100 µl into 0.5 ml sterile micro-centrifuge tubes and store in -20°C, Non-frost-free freezer

bLH

1. Dilute (1) 25-unit vial of bLH with 12.5 ml of TCM199
2. Aliquot 100 µl into 0.5 ml sterile micro-centrifuge tubes and store in -20°C, Non-frost-free freezer

Procedure:

1. Combine above reagents into a 14 ml sterile polystyrene snap-cap tube
2. Syringe filter using a 10 ml Norm-Ject syringe and a 0.25 µm syringe filter (Acrodisc)
3. Store with cap loose in 5% CO₂ in air 38.5°C in humidified incubator. Discard unused portion after 12 hours

J

FEED
(Producers CO-OP, College Station, TX)

INGREDIENTS:

1. Wheat midds
2. Corn
3. Bakery product
4. Cottonseed hulls
5. Soybean hulls
6. Cottonseed meal
7. Liquid binder
8. Ground lime
9. Rice bran
10. Ammonium sulfate
11. Salt mixing
12. Beef vitamins
13. Beef trace minerals
14. Rumensin

FEED:

Nutrient Name	Units	Actual	Dry Matter	Minimum	Maximum
Weight	Lbs	1.0000	1.0000	1.0000	1.0000
Protein	%	12.0000	13.492	12.0000	
Fat	%	4.1266	4.6396	2.5000	4.5000
Fiber	%	12.8176	14.4113	12.0000	20.0000
Calc	%	0.6925	0.7785	0.5000	0.7500
Phos	%	0.4522	0.5085	0.4000	
AV P	%	0.1580	0.1777		
TDN	%	70.7396	79.5351	70.5000	
NE M	MCal/Cwt	81.2352	91.3357	78.5000	
NE G	MCal/Cwt	53.3950	60.0339	53.0000	
NPN	%	0.9750	1.0962		1.5000
R.F.	%	22.0000	24.7354	20.0000	35.0000
Salt	%	0.9348	1.0511	0.5000	1.0000
Potassium	%	0.7391	0.831	0.6800	
Sulf	%	0.3251	0.3655		
Magn	%	0.2429	0.2731		
Mang	ppm	124.6946	140.1986		
Iron	ppm	134.5410	151.2694		
Copr	ppm	23.1328	26.009		
Cobt	ppm	0.8124	0.9134		
Zinc	ppm	89.7947	100.9594		
Iodn	ppm	1.3750	1.546		
Seln	ppm	0.3816	0.429		
Vit A	IU/LB	5506.9220	6,191.63	2000.0000	

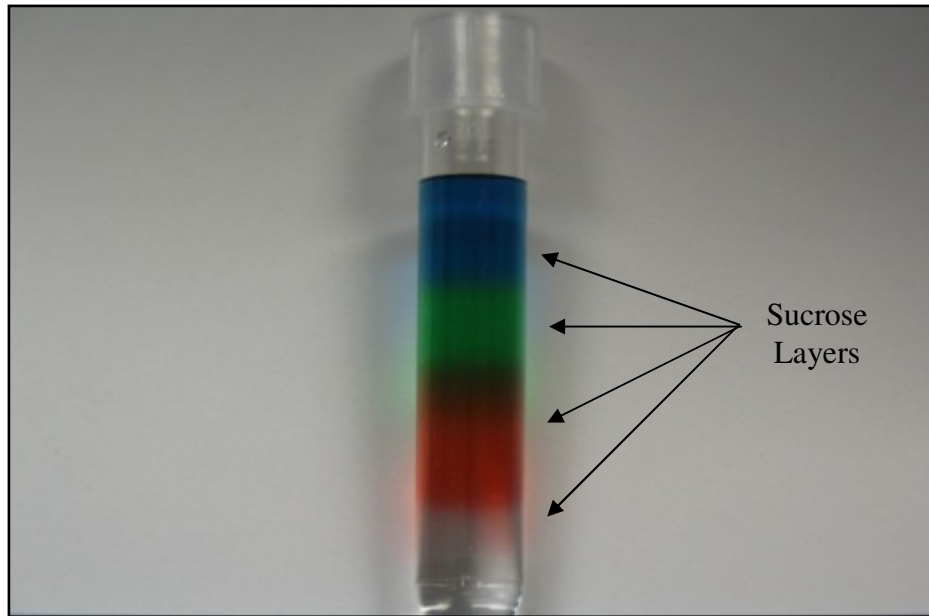
Vit E	IU/LB	22.8095	25.6456		
Zn:Cu	ppm	3.8817	3.8817		
Sodium	%	0.4107	0.4618		
D>M.	%	88.9414	88.9414		
Ca:P	Ca:P	1.5312	1.5312	1.5000	2.0000
Monensin	g/lb	0.0150	0.0169		
VIT A Added	IU/LB	4000.0000	4,497.35		
VIT E Added	IU/LB	5.0000	5.6217		
VIT D Added	IU/LB	1000.0000	1,124.34		
Seln Added	ppm	0.0375	0.0422		

K



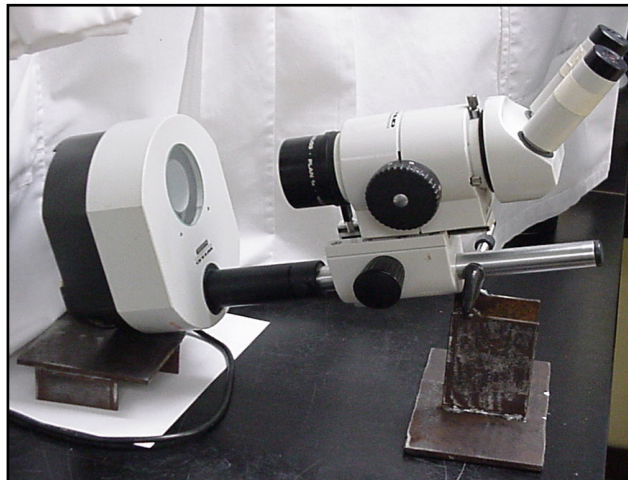
Porcine (A) and bovine (B) ovaries obtained from a local abattoir prior to follicle aspiration.

L



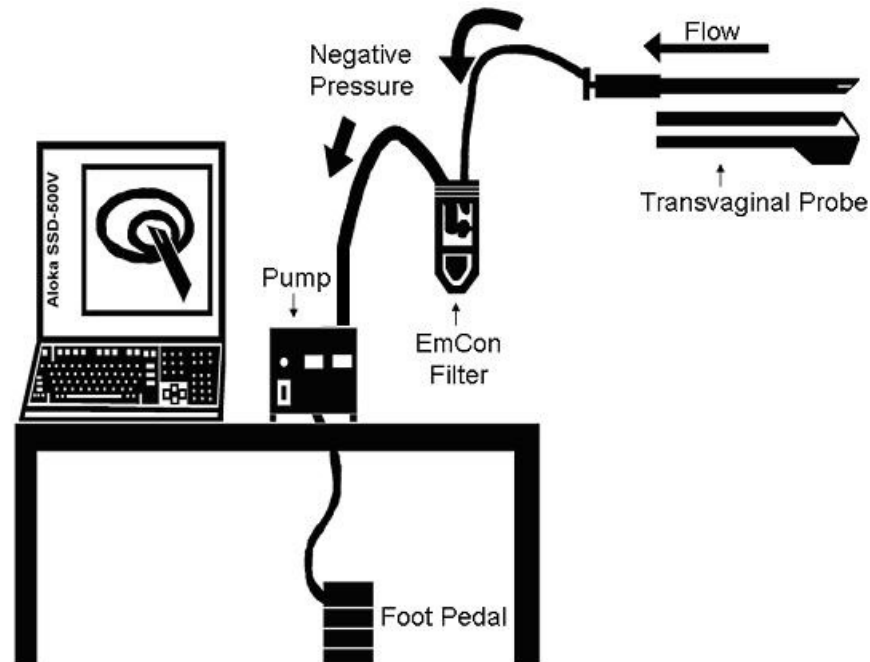
Buoyant density gradient layered from 14 to 30% sucrose gradient. Color was added to evaluate the stability of the gradient.

M



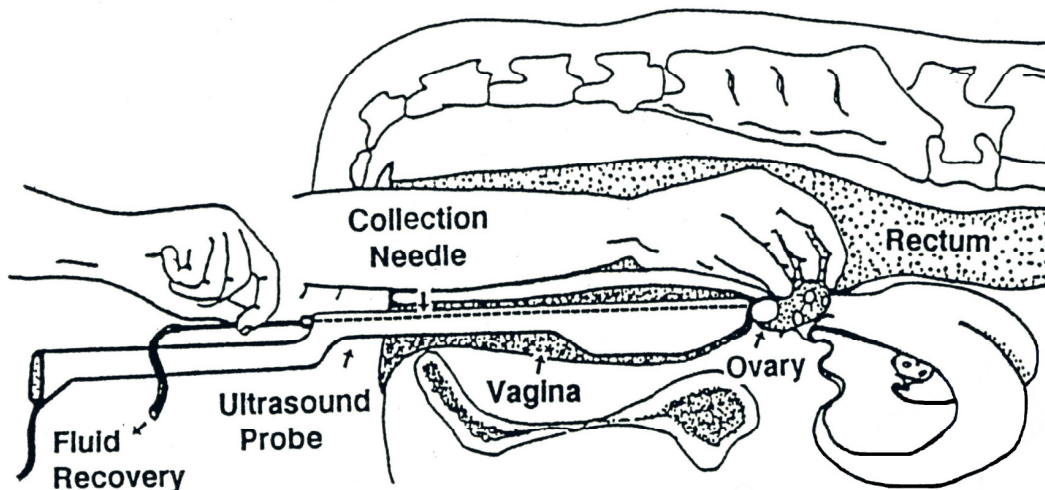
Pictures of horizontal microscopes used to evaluate M-II oocytes in sucrose buoyant density concentration.

N



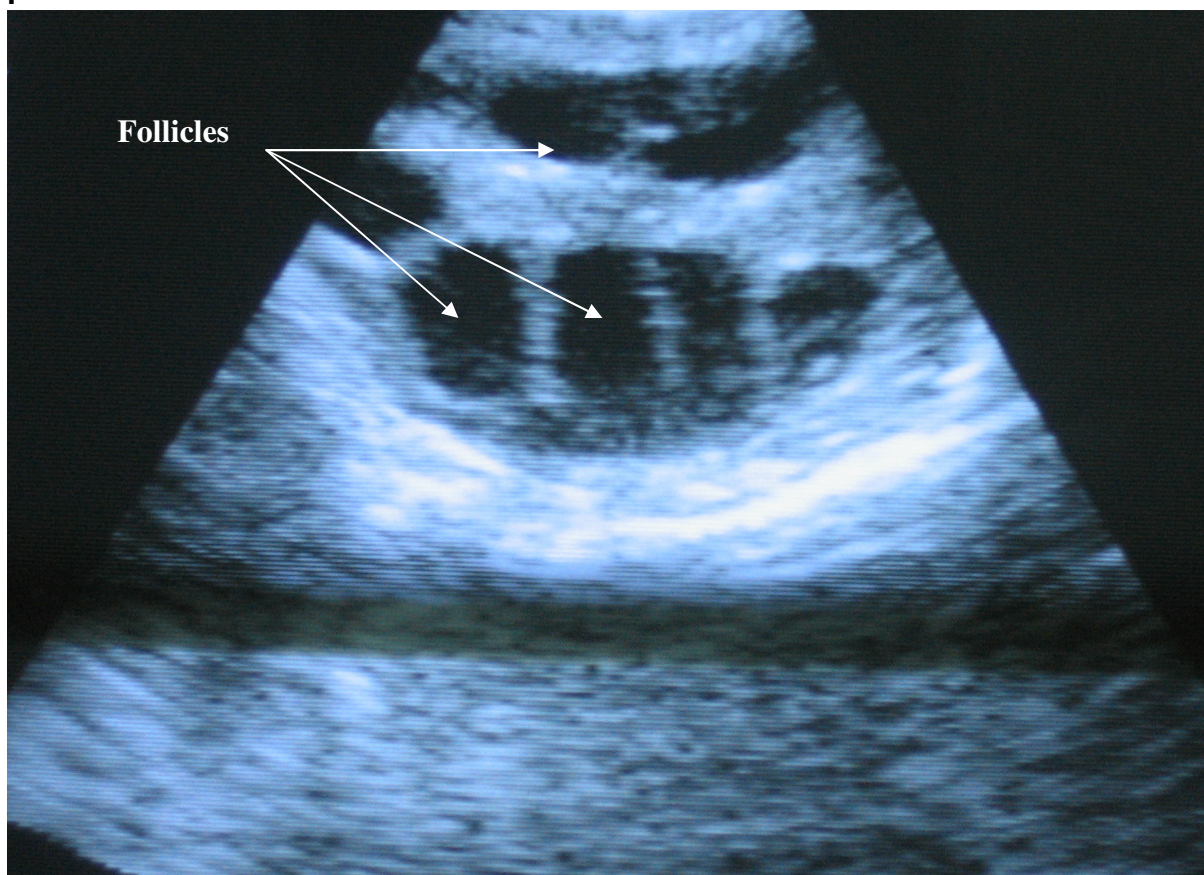
Schematic drawing of tubing and pump configuration used for transvaginal ultrasound-guided oocyte aspirations (TUGA) of donor oocytes. (Drawing based on original by Dr. J. Broussard).

O



Schematic drawing of the hand and ovary placement during TUGA procedures (Illustration by Dr. A. Meintjes).

P



Follicles from a stimulated Brahman donor shown on the Aloka SSD-500V monitor just prior to TUGA procedures.

VITA

Casey Bryant Ballard was born to Larry and Linda Ballard in Texarkana, Texas, on July 25, 1980. He was raised on a purebred Beefmaster ranch located in Atlanta, Texas, where he developed an understanding and love for animals and the livestock industry. He graduated from Atlanta High School in Atlanta, Texas, in May, 1999.

He received a bachelor of science in agriculture, with a major in agribusiness, from Texas A&M University in May, 2004. He entered the graduate school at Louisiana State University where he completed 2 years of study and a 5 month work study at Ovagenix (Bryan, Texas).